# THE EFFECTS OF PRENATAL TRANSPORTATION ON POSTNATAL ENDOCRINE AND IMMUNE FUNCTION IN BRAHMAN BEEF CALVES

A Thesis

by

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ABSTRACT

Prenatal stressors have been reported to affect postnatal cognitive, metabolic, reproductive and immune functions. This study examined immune indices and function in Brahman calves prenatally stressed by transportation of their dams on d 60, 80, 100, 120 and 140 ± 5 d of gestation. Based on assessment of cow's temperament and their reactions to repeated transportation it was evident that temperamental cows displayed greater pre-transport cortisol ($P < 0.0001$) and glucose ($P < 0.03$) concentrations, and habituated slower to the stressor compared to cows of calm and intermediate temperament. Serum concentration of cortisol at birth was greater ($P < 0.03$) in prenatally stressed versus control calves. Total and differential white blood cell counts and serum cortisol concentration in calves from birth through the age of weaning were determined. We identified a sexual dimorphism in neutrophil cell counts at birth ($P = 0.0506$) and cortisol concentration ($P < 0.02$) beginning at 14 d of age, with females having greater amounts of both. Whether weaning stress differentially affected cell counts, cortisol concentrations and neutrophil function of prenatally stressed and control male calves was examined. At 2 d post weaning, all calves had increased cortisol concentration ($P < 0.0001$) and neutrophil cell counts ($P < 0.0001$). However, *in vitro* production of reactive oxidative species by neutrophils was decreased ($P = 0.0002$) 2 d post weaning. Moreover, prenatally stressed calves demonstrated a larger ($P = 0.0203$) decrease in their immune function relative to control calves at 2 d post-weaning. Importantly, prenatally stressed calves took longer than controls to recover from the

weaning stress. Additional studies are needed to clarify if prenatally stressed calves are more susceptible than control calves to pathogens during the post weaning period. Management practices to improve animal welfare and livestock production may need modification if follow-up studies demonstrate that prenatal stress also affects reproductive development, growth, performance and meat quality.

# DEDICATION

To my Mom and Dad who have always been there for me and encouraged me to do my best, I am always and will be forever grateful. To my grandma who always wanted to see me succeed, I know she would be proud if she was here. To my family, I love you all and would not be the person I am without all of your love and guidance.

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# LITERATURE REVIEW

## Introduction

Throughout life, all animals encounter various internal or external challenges that elicit a stress response in the animal and its systems. The biomedical implication of the term "stress" was initially applied by Dr. Han Selye when he defined stress as the "general alarm reaction" of an organism to a nonspecific nocuous agent (Selye, 1936). Physiologists now consider the nonspecific agent that causes a physiological response in the animal to be a stimulus, termed a "stressor". Several types of stressors exist, which include environmental, physical, social, nutritional, and even the body's reaction during a diseased state. Stress can be acute, considered to last mere minutes or hours, or chronic, typically defined as continuing for multiple hours per day for weeks or months at a time (Dhabhar, 2009). Selye also defined an organism's reactions to overcome stress as the "general adaptation syndrome", noting its effects on the immune system through his observations of reduced thymus size (Selye, 1936, 1973). Stress causes alterations in an animal and its systems by disrupting homeostasis, defined as the coordinated physiological reactions that maintain the body's steady state (Cannon, 1929).

Once an animal is stressed, its body makes attempts to alleviate stress in various ways. Immediately after encountering a stressor, physical and behavioral adaptions

occur redirecting the animal's energy to where it is needed. Adaptations include heightened arousal and cognition, increased heart rate and respiratory rate, arterial blood pressure, and body temperature (Chrousos and Gold, 1992; Sapolsky et al., 2000; Chrousos, 2009). Additionally, metabolic changes in the form of increased gluconeogenesis and lipolysis take place redirecting nutrients and energy toward the central nervous system (CNS) (Sapolsky et al., 2000; Chrousos, 2006). Simultaneously, functions unnecessary in coping with the stressor are inhibited such as appetite, reproduction, growth, digestion, and immunity (Chrousos and Gold, 1992; Sapolsky et al., 2000; Chrousos, 2006). These resulting changes cause either transient or long term consequences in the animal.

The term allostasis is used to describe the chronic deviation from homeostasis resulting in insufficient/repeated adaptive responses, whereby the individual survives, but deals with negative consequences (McEwen and Wingfield, 2003; Charmandari et al., 2005; McEwen and Wingfield, 2010). McEwen and Wingfield (2003, 2010) further go on to classify allostatic load as the daily and seasonal activities an animal faces that are necessary for its survival, reproduction and any additional unforeseen obstacles the animal may encounter. When the allostatic load on an individual becomes too great, as often happens in cases of long-term chronic stress, increased occurrences of pathologies are observed.

The ongoing research on stress aims to elucidate both the mechanisms and outcomes to the alterations stress causes on an animal and its systems. Currently, there is evidence supporting both beneficial and deleterious responses to stress. As each

animal has its own genetic background and environmental experiences and each situation is different; responses to each situation an animal may face will vary accordingly.

The damaging effects of stress include changes in behavior, growth, metabolic, immune, endocrine, and reproductive function. Animals have been shown to display reduced coping abilities after they have been subjected to chronic stressors for long periods of time (de Kloet et al., 2005). Decreased cytotoxic ability of T cells along with decreases in interferon-γ (IFN-γ) production were observed in medical students during examination periods compared to 1 month before examinations (Glaser et al., 1987). Increased incidences of various pathologies have been associated with prolonged or chronic stress. Neuronal degeneration occurs after prolonged exposure to high concentrations of endogenous glucocorticoids (GC) (Uno et al., 1989). Vervet monkeys that endured continuous social stress throughout their life displayed increased hippocampal neuronal loss along with hyperplasia of the zona fasciculata of their adrenal cortices (Uno et al., 1989).

Not all stress is maladaptive. For instance, acute stress causes a pro-inflammatory response resulting in the production of cytokines and readying the immune system to respond to the impending threat to homeostasis. Experiencing acute stress prior to surgery or vaccination can aid in immunoprotection through the body's recruitment and redistribution of leukocytes to the site of surgery or immune activation (Dhabhar and McEwen, 1997; Dhabhar and Viswanathan, 2005; Viswanathan and Dhabhar, 2005). Chronic stress has been shown to help end the inflammatory response

by decreasing production of cytokines, chemokines, and other factors that would initiate damage to the body. Glucocorticoid therapy has been and still is employed in an array of medical treatments for several autoimmune mediated diseases including those for arthritis and Addison's disease. The use of glucocorticoids for disease treatment came from studies in the late 1940s in rheumatoid arthritis patients given an adrenal cortex compound, 17-hydroxy-11-hydrocorticosterone, and subsequently, ACTH (Hench and Kendall, 1949).

**Stress and the Involvement of the HPA Axis**

One of the major components of the stress response is the hypothalamic-pituitary-adrenal axis (HPAA). The HPAA receives and responds to signals about the body's current state. Once the HPAA is activated, a series of events follow aiming to relieve the perceived stress on the animal's system. The hypothalamus receives signals about stressors from the body and in response neurosecretory neurons in the hypothalamic paraventricular nucleus (PVN) secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (VP) (Brown, 1994). Originally discovered in sheep, CRH is a 41 amino acid peptide and used to be named CRF (Vale, 1981). Together these peptide neurohormones travel through the hypophyseal portal vessels where they act on corticotrophes in the anterior pituitary. This mode of hormone travel and subsequent stimulation was elucidated through experiments using electrical stimulation and lesions of the hypothalamus in rabbits and rats (De Groot and Harris, 1950).

Corticotrophes of the anterior pituitary produce a pre-cursor protein known as proopiomelanocortin (POMC), which can be cleaved into several distinctly and

biologically active peptides (Nakanishi et al., 1979; Herbert, 1981; Brown, 1994). Two important products of POMC cleavage are adrenocorticotrophic hormone (ACTH), and β-endorphin, an endogenous opioid (Blalock, 1994; Brown, 1994). After being released from the anterior pituitary, ACTH travels through the circulation to the adrenal glands stimulating the adrenal cortex's production and release of glucocorticoids (GC) (Axelrod and Reisine, 1984; Brown, 1994). Both VP and CRH act synergistically to stimulate each other in a positive feedback loop.

The GCs act on both glucocorticoid receptors (GR) and mineralcorticoid receptors (MR) in their target tissues modifying adrenal cortex GC production through their regulation of gene expression in the CNS, hypothalamus and pituitary (Evans, 1989; Burdick et al., 2011c; Herman et al., 2012). Once bound to the GR, GCs will cause the GR to migrate into the nucleus from the cytoplasm where it will bind to glucocorticoid response elements and cause changes in gene expression (Jones, 2012). Measuring concentrations of GCs (cortisol in ruminants and humans; corticosterone in rats and mice) allows researchers to quantitatively measure an animal's response to stress. The GCs also help regulate the HPA axis' response by negative feedback mechanisms; when GCs are high, CRH release is reduced. This is because high concentrations of GCs are able to block the hypothalamic secretion of CRH. A prolonged or excess exposure to high concentrations of GCs can alter the HPA's response to GCs through alterations in the expression of GR (Cottrell and Seckl, 2009). The degree of expression of GR in the body can be changed in response to cortisol concentrations. Endogenous GCs preferentially bind MRs, but during the stress

response, the excessive levels of GCs bind GRs instead (Evans, 1989; Burdick et al., 2011c). This leads to detrimental consequences in the animal.

The other major regulator of the HPA is the sympathetic nervous system (SNS). This part of the nervous system is responsible for the acute phase response. After encountering a stressor, an animal's body will activate the sympathetic nervous system which is responsible for the "fight or flight" response. Pre-ganglionic nerve fibers in the adrenal medulla secret acetylcholine (AcH), which triggers the production and release of the catecholamines, epinephrine (E), norepinephrine (NE), and dopamine from the adrenal gland's medullary components (Verbrugghe et al., 2012). The catecholamines, E and NE bind to β-adrenergic receptors in lymphoid cells and activate the cAMP pathway promoting a proinflammatory response through their production of cytokines (Verbrugghe et al., 2012).

Subsequently, production of inflammatory cytokines interleukin-6 (IL-6), Il-1, and transforming neurotrophic factor - α (TNF-α) act to further stimulate the HPA to increase its secretion of CRH and thus GCs (Smoak and Cidlowski, 2004). High concentrations of GCs cause immunosuppression through their inhibition of cytokine production needed for the regulation of immunoglobulin synthesis and release. The expression of the proinflammatory cytokines IL-1β, IL-1α IL-8, lymphotoxin-β, IFN-α and IFN-β are known to be down-regulated by glucocorticoids; aiding in the reduction of an inflammatory response (Galon et al., 2002). Similarly, the expressions of such cytokines as transforming growth factor (TGF)-β3, IL-10, and IL-10R, act to stem the production of proinflammatory cytokines, are up-regulated (Galon et al., 2002). This

down-regulation of cytokines is seen as protective and is thought to prevent an overshoot of the inflammatory response, which would cause damage to the animal's tissues (Munck et al., 1984; Sapolsky et al., 2000).

**Prenatal Stress: Gestation and the Effects of Stress**

Gestation is a critical time of development for any growing animal. It is when all of an animal's organs and organ systems develop. Prenatal stress has been studied in a range of animals with varied effects being dependent upon the stage of gestation, either early or late, when the stressor is applied (Couret et al., 2009). If a female experiences increased stress during the early stages of gestation, it can lead to an increased chance of pregnancy loss. Higher concentrations of cortisol in women were found to be associated with pregnancy loss during the first 3 weeks after conception (Nepomnaschy et al., 2006). Thermal stress along with intensive management has been implicated in pregnancy losses in dairy cattle (Zobel et al., 2011). Additionally, the consequences of prenatal stress may vary based upon the sex of the fetus and the species employed in the study (Matthews, 2002).

Recent research has shown a fetus can be affected in utero by the mother's nutrition (Wu et al., 2004) and the various types of stressors she faces. Increased risk of adult development of ischeamic heart disease, cardiovascular disease, insulin resistance, and metabolic disease are associated with low birth weights, reduced/poor fetal and infant growth and likely due to changes in the HPAA activity of the offspring (Barker et al., 1989; Phillips et al., 1998; Gluckman et al., 2008). The effect on the fetus/offspring resulting from prenatal stress has been termed developmental programming.

Developmental plasticity is used to describe the way in which adaptations to the environment can result in one genotype having various physiological or morphological states (Barker, 2004). The effects stress has on a mother's offspring aren't fully realized until later in life. Scientists are just beginning to understand the impacts prenatal stress has on the developing offspring.

Prenatal stress is especially important in animal production because prenatal (Zobel et al., 2011) and postnatal (Tuchscherer et al., 2002) animal losses reduce a producer's profit and represent continuous problems affecting the livestock production industry. Since prenatal stress has been shown to alter immune function of the developing offspring (Tuchscherer et al., 2002; Couret et al., 2009), this can lead to even larger consequences and concerns for welfare of neonates in the animal production industry. Reductions in stress, along with improvements to their health and welfare can lead to increases in animal health and well-being, quality and reproductive ability; all of which add to the producers' profits. Producers want to obtain the most out of their investments, so researching ways to improve management strategies or breeding programs can be beneficial to increasing animal welfare, profitability and performance (meat quality and growth rates) of production animals.

A considerable amount of research has been done in rats and other lab animals on how different types of prenatal stressors affect the offspring. Pregnant rats subjected to stress by either inescapable electric foot shock or psychological stress during days 15-21 of gestation, delivered offspring with lower levels of IgGs on postnatal day 0 compared to pups from control rats (Sobrian et al., 1992). Restraint stress of pregnant rats under

bright lights was observed to decrease NK cell cytotoxicity in male juveniles and conversely enhanced it in both sexes as adults (Klein and Rager, 1995). Studies in rats have shown prenatal endotoxin exposure caused elevated corticosterone concentrations in the mothers and subsequently reduced basal and post immune responses following a lipopolysaccharide (LPS) challenge preweaning in their offspring compared to controls (Hodyl et al., 2007). Particularly, lymphocytes, monocytes, and Il-β were reduced in prenatally exposed rats (Hodyl et al., 2007). Similarly, Coe and his colleagues found blood cells from prenatally stressed monkeys at 2 yr of age to have reduced production of the cytokines TNFα and Il-6 after stimulation with both LPS and dexamethasone (DEX) (Coe et al., 2002). Their experiments demonstrated lasting effects of prenatal stress past those of the neonate and infant.

Investigations in the realm of prenatal stress have been lacking with regard to production animals. However, the effects of prenatal stress have recently begun to be reported in pigs (Tuchscherer et al., 2002; Couret et al., 2009), cattle (Lay et al., 1997a; Lay et al., 1997b), goats (Duvaux-Ponter et al., 2003; Roussel et al., 2005), and sheep (Roussel et al., 2004). Experiments in prenatally stressed pigs showed decreased immune function in the piglets and increased neonatal deaths (Tuchscherer et al., 2002). Prenatally stressed piglets displayed significantly reduced concentrations of IgG during the first 3 days of life, and suppressed proliferation of lymphocytes to Concanavalin-A (ConA) and LPS during the first 5 weeks of life (Tuchscherer et al., 2002).

Prenatal stress has widespread consequences for the HPA axis. Studies in rats, guinea pigs, and cattle have shown prenatal stress alters the abilities of their offspring to

respond to stressors (Lay et al., 1997b; Weinstock, 1997; Weinstock et al., 1998; Kapoor and Matthews, 2005). Prenatally stressed rats' offspring displayed higher cortisol concentrations during basal and after foot shocks than control animals (Weinstock et al., 1998). Prenatal exposure to strobe lights for 2 h during d 50, 51, and 52 (PS50) or d 60, 61, and 62 (PS60) of gestation led to increased basal plasma cortisol in the male PS50 guinea pigs and elevated cortisol responses to an ACTH challenge in PS60 guinea pigs (Kapoor and Matthews, 2005). Similarly, in cattle, 10 day old and 150 day old offspring of cows that were repeatedly transported during gestation displayed greater plasma cortisol concentrations and decreased cortisol clearance rates than control calves during restraint and after cortisol injections (Lay et al., 1997b). Another study by the same group, showed increases in pituitary, heart, and body weights of fetal calves resulted from prenatal stress (Lay et al., 1997a).

Offspring of prenatally stressed animals also are known to display problems with reproductive functions (Herrenkohl, 1979; Kapoor and Matthews, 2005). Rats whose mothers were stressed prenatally by light delivered fewer offspring, had decreased litter weights, and had increases in pregnancy and neonatal losses compared to unstressed controls (Herrenkohl, 1979). This effect was not due to changes in milk production or care given to the rat pups as another experiment used cross-fostering to raise the prenatally stressed rats, and found similar results (Herrenkohl, 1979). Male guinea pigs of prenatally stressed mothers (d 50-52 of gestation) displayed decreased testosterone concentrations relative to controls (Kapoor and Matthews, 2005).

## The Immune System

The immune system of animals consists of both innate and acquired immunity. Both arms of the immune system work together in a coordinated effort to protect the body from infection and neutralize any pathogens it encounters. The innate immune system is responsible for an animal's immediate response to what the body considers foreign. It is also responsible for activating the acquired immune system to produce cells necessary to fight an invading pathogen or microbe. The adaptive or acquired immune system is the second branch of the immune system and is divided into two parts, cell-mediated immunity, governed by the T cells and humoral immunity which is directed by B cells. Additionally, the adaptive immune system is responsible for the secondary response, which is able to respond to a wider array of pathogens and typically takes days to take effect.

### *Innate Immune System*

As the primary protection system of the body, the innate system is always active, quietly running its surveillance in the background until it finds an "intruder" and a reason to activate and attack. There are both physical and chemical barriers and cellular parts that make up the innate immune system. On the physical level, there is skin, which protects the organism from microbial pathogens (Elias, 2007), mucosal secretions from the mucosal layers, the acidic pH of the stomach which serves not only to digest food but can inactivate certain bacteria as well (Abbas and Lichtman, 2011; Murphy et al., 2012). The innate immune system includes three types of cells: phagocytic cells, natural killer cells, and cells that release inflammatory mediators. Phagocytic cells include

neutrophils, macrophages, monocytes, and dendritic cells. These cells are responsible for recognizing a conserved set of molecules on microbes in the body known as pathogen-associated molecular patterns (PAMPs) (Palm and Medzhitov, 2009; Abbas and Lichtman, 2011; Murphy et al., 2012).

The germ-line encoded receptors responsible for recognizing PAMPs are called the pattern recognition receptors (PRRs) and include such receptors as CD14, DEC205, collectins, toll like receptors (TLRs), and complement receptors (Medzhitov and Janeway, 1997). After TLRs on phagocytes recognize a microbe's PAMPs, the phagocytes ingest the microbe into their vesicles where they kill them (Abbas and Lichtman, 2011; Murphy et al., 2012). Dendritic cells and macrophages serve as antigen presenting cells (APCs). After digestion of foreign antigens, they display proteins from the antigen on their cellular surface for presentation to the T cells (Murphy et al., 2012). The T cells and the adaptive immune system are thus activated by the APCs in the lymph nodes (Abbas and Lichtman, 2011). Additionally, the innate immune system possesses a set of soluble plasma proteins responsible for opsonizing or marking cells for phagocytosis collectively known as the complement system (Murphy et al., 2012). The complement system also participates in enhancing the responses of the B and T cells to antigens.

Natural killer cells (NKCs) attack and kill cells infected by pathogens or abnormal cells they come across in the body. They do so by recognizing the levels of major histocompatibility complex (MHC) class I expression on cells of the body and target cells abnormally expressing MHC class I proteins; which is commonly the case in

infected cells or transplanted cells which are foreign to the body (Boysen and Storset, 2009). The NKCs destroy infected cells through their release of granules such as perforins or granzymes, which act to perforate a hole into the cell wall of the target cell causing it to lyse (Boysen and Storset, 2009). Another method of microbial killing by NKCs is through their initiation of the Fas receptor on the infected cell, leading to apoptosis, or programmed cell death (Boysen and Storset, 2009). Macrophages and monocytes initiate the innate immune response with their production of cytokines, IL-1, IL-6, and TNF-α (Blalock, 1989; Carroll and Forsberg, 2007). Monocytes become macrophages upon their migration from the blood into the tissue.

Some of the first cells to migrate to the site of infection or inflammation are the neutrophils or polymorphonuclear leukocytes (PMN). Neutrophils represent the major cell type in the differential leukocyte counts of adult cats and dogs (Moreira da Silva et al., 1994). However, in adult cattle, lymphocytes are the predominant cell type produced, with neutrophils constituting only 25% of the total leukocyte count (Moreira da Silva et al., 1994; Paape et al., 2003). Neutrophils are short lived cells that typically survive for about 6-7 h in the circulation, before undergoing apoptosis (Amulic et al., 2012). Furthermore, neutrophils produce a respiratory burst, which results in the production or release of reactive oxidation species (ROS) capable of killing many types of bacteria. Through the process of degranulation and ROS production neutrophils are rapidly killed. This is why they represent the first line of defense and are considered essential for survival.

*Adaptive or Acquired Immune system*

Acquired immunity comes from an animal being exposed to pathogens or infectious agents. The adaptive immune system is unique as it is only found in vertebrates and it possesses the ability to create memory cells which prepare the body for future encounters with the same antigens (Medzhitov and Janeway, 1997; Murphy et al., 2012). Memory cells allow for a quicker and stronger secondary response that is characteristic of the adaptive immune system (Abbas and Lichtman, 2011; Murphy et al., 2012). After being activated, the animal's body creates antibodies to the antigens it encounters. The acquired immune system consists of lymphocytes which are specialized white blood cells (WBC). The lymphocytes include both the B and T cells which originate in the bone marrow and mature in the bone marrow and thymus, respectively (Abbas and Lichtman, 2011). Prior to their maturation, pre-lymphocytes lack receptors for either B or T cells; however, once they are directed toward one cell line or the other, cell markers for either B or T cells will develop. Lymphocytes proliferate in response to pathogens, cytokines and chemokines secreted by various components of the immune system. Both B and T cells work together to help eliminate pathogens. T cells are responsible for producing cytokines, recruiting other cells to areas of infection or inflammation and killing infected cells and microbes. The B lymphocytes are responsible for the production and release of antibodies or immunoglobulins (Igs) and will alter the isotypes of Igs produced in response to various cytokines (Abbas and Lichtman, 2011; Murphy et al., 2012).

The T lymphocytes consist of the $CD4^+$ (helper T, TH) cells and the $CD8^+$ (cytotoxic or cytolytic T cells, CTL), named for the proteins expressed on the cellular surface (Murphy et al., 2012). The TH cells can be further divided into 3 subsets, TH1, TH2, and recently, TH17. Each is identified by the different cytokines and transcription factors that initiate their differentiation and also by the cytokines they produce (Wei et al., 2007). Furthermore, the cytokines stimulating differentiation of each type of TH cell act to inhibit the differentiation of the other TH effector subsets. The TH1 cells are produced with stimulation from IFN-γ, IL-12, and the transcription factors STAT4 and T-bet (T-box expressed within T cell) (Wei et al., 2007; Bonilla and Oettgen, 2010). This subset is known for their ability to initiate activation of phagocytic cells, NKCs, and CTLs resulting from their release of IFN-γ and IL-2 (Bonilla and Oettgen, 2010). Conversely, TH2 cells are dependent on the expression of GATA-3, STAT-6, and the cytokine IL-4 (Bonilla and Oettgen, 2010; Bi and Yang, 2012). After development, TH2 cells produce IL-4, IL-5, IL-10 and IL-13, which are important against parasitic immune responses, triggering eosinophil development and increasing antibody production (Bonilla and Oettgen, 2010). It is thought that basophils may have a role in the direction of TH cells toward the TH2 cell type (Schroeder, 2009). Development of the recently discovered TH17 cells is triggered by retinoic acid receptor related orphan receptor γt (RORγt), RORα, STAT3 and the presence of TGF-β, IL-6 and IL-23 (Bi and Yang, 2012). In turn TH17 cells create IL-21, and a family of IL-17 cytokines (IL-17A through IL-17F) (Bi and Yang, 2012).

The antibody repertoire of B cells includes IgA, IgD, IgE, IgG, and IgM, each with a specific role in the body's defense system. IgM is the largest and first of the Igs to be produced during the primary response to an infection or inflammation. IgG is produced in 2 forms, IgG1 and IgG2, which have roles in neonatal immunity as they are transferred across the placenta and are typically responsible for aiding in the immune response to pathogens (Gapper et al., 2007). In cattle IgG1 is the predominant IgG in colostrum, while IgG2 is mostly found in the serum of adult cattle (Gapper et al., 2007). In cattle, IgG2 was shown to facilitate phagocytosis by both monocytes and neutrophils of IgG2 antibody coated erythrocytes, while IgG1 was unable to stimulate phagocytosis (McGuire et al., 1979). Conversely, the same study also observed both IgG1 and IgG2 to stimulate phagocytic ability of monocytes when cultured with erythrocytes for 7 days (McGuire et al., 1979). IgA is responsible for mucosal immunity as it is secreted from the mucosal epithelial layers. The production of IgE is regulated by the cytokines, IL-4 and IL-13. Some of IgE's characteristics include its role in allergic reactions and immunity against helminthes and parasitic infections. It is the antibody produced in the smallest quantities and is unable to cross the placenta during pregnancy.

*Hematopoiesis*

Development of both the red and white blood cells begins in the bone marrow with the self-renewing pluripotent stem cells (PSCs ) also called the colony-forming units-spleen (CFU-S) that are formed during the embryonic stage (Moreira da Silva et al., 1994). Stem cells will then differentiate either along the lymphoid lineage where they will become B and T lymphocytes, or the myeloid lineage. The myeloid cell line

includes erythrocytes or red blood cells (RBCs), platelets, monocytes, macrophages, and the granulocytes which include neutrophils, basophils and eosinophils (Moreira da Silva et al., 1994). Macrophages are believed to be the default cell generated from the myeloid lineage as granulocytes require various transcription factors, proteins, and receptors to be expressed for their formation (Cowburn et al., 2008).

*Neutrophil Formation*

Granulopoiesis or the formation of granulocytes results in development and production of basophils, eosinophils, and neutrophils, each triggered by various stimulating factors. The formation of neutrophils takes on average 6 d to complete and involves several cellular stages, each characterized by the shape of their nucleus, size and types of granules produced (Paape et al., 2003). The stages of neutrophil formation in developmental order are myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and the segemented neutrophil which is a mature neutrophil or polymorphonuclear leukocyte (PMN) (Bainton et al., 1971). The stages of neutrophil maturation each take place in three different compartments of the bone marrow. The mitotic pool consists of the proliferating myeloblasts, promyelocytes and myelocytes; the non-mitotic pool which comprises the maturing metamyelocytes and band cells; and finally the storage pool contains mature PMNs and some band cells (Moreira da Silva et al., 1994; Van Merris et al., 2002).

The myeloblast is the first cell type formed; is the smallest and contains mainly cytoplasm and lacks granules. The myeloblast is followed by the promyelocyte and is characterized by its round nucleus, large size, and presence of primary or azurophilic

granules (Bainton et al., 1971). Azurophilic granules are peroxidase-positive due to their containing myeloperoxidase (MPO) and demonstrate peroxidatic activity upon stimulation with hydrogen peroxide (Amulic et al., 2012). They are responsible for killing bacteria. The first expression of the Fc-$IgG_2$ receptor was detected during the myeloblast and promyelocyte stages and was shown to increase in mature PMNs (Van Merris et al., 2002).

The next cell stage manufactured is the myelocyte; known for its indented nucleus, noticeable golgi complex, despite its lack of peroxidase reactivity and assorted granules (Bainton et al., 1971). During the myelocyte stage, secondary or specific granules are formed. They are smaller in comparison to the azurophilic granules, and lack MPO. Instead they primarily contain the glycoprotein lactorferrin (Amulic et al., 2012). An additional granule type has been shown to exist in cattle, sheep and goats. It is larger than the other granule types, lacks peroxidase, and is the predominate granule in adult cattle (Gennaro et al., 1983; Paape et al., 2003). It is believed to function in oxygen-independent killing of bacteria as in the respiratory burst that occurs in neutrophils.

Metamyelocytes represent the next stage in PMN development and are identified by their large azurophilic granules, non-active golgi complex, and accretion of glycogen (Moreira da Silva et al., 1994). Next in the maturation process is the band cell with its smooth nuclear membrane that differentiates it from mature PMN with a more indented and irregular nuclear membrane (Moreira da Silva et al., 1994).

Once mature, PMN are ready to leave the bone marrow and enter the circulation, where they typically circulate for about 6-7 hours (Amulic et al., 2012). In response to cytokines and chemokines, they are directed to migrate into tissues during infection or inflammation by chemotaxis. Without stimulation from chemoattractants, they will undergo apoptosis, or a programmed cell death, which is thought to prevent damage from the release of their toxic components into the organism's tissue (Amulic et al., 2012).

As neutrophils endogenously flow through the blood they weakly adhere to the vascular endothelium with the aid of calcium dependent adhesion molecules, called selectins (Kelly et al., 2007). There are three known selectins, E-selectin (CD62E), L-selectin (CD62L), and P-selectin (CD62P), expressed by endothelial cells, leukocytes, and platelets and endothelial cells, respectively (Kelly et al., 2007; Weiss and Walcheck, 2008). Out of the three selectins, L-selectin has the most widespread effects on neutrophil capture, adhesion and accumulation on the endothelium. Specifically, it is the up or down regulation of L-selectin on leukocytes that can impact the recruitment of mature PMN from the bone marrow and subsequently their adhesions to the endothelium (Cavalcanti et al., 2007; Weiss and Walcheck, 2008). Interestingly, glucocorticoids and DEX have been shown to regulate the expression of L-selectin, under both endogenous and inflammatory conditions (Burton et al., 1995). Bovine neutrophils treated with DEX for 4 h displayed a down-regulation of CD62L mRNA, which was reversed with treatment of the cortisol antagonist, RU486 (Weber et al., 2007). Furthermore,

adrenalectomized rats exhibited an increased expression of L-selectin on peripheral neutrophils resulting in greater concentrations of band PMNs (Cavalcanti et al., 2006).

In response to infectious or inflammatory stimuli, such as TNF-α, IL-1, and IL-17, endothelial cells will activate and initiate the activation of neutrophils through their production of IL-8 and MIP-2 (Borregaard, 2010). Upon activation, neutrophils become firmly attached to the endothelium to prepare for diapedesis or migration into tissues with the aid of a family of transmembrane glycoproteins called β2 (CD18) integrins (Weiss and Walcheck, 2008; Amulic et al., 2012). The β2-integrins are made up of an α-subunit (CD11a or CD11b) and a β-subunit (CD18) and include lymphocyte function-associated antigen (LFA-1 or CD11a/CD18) and MAC-1 (CD11b/CD18) which bind to ligands on the epithelial surface, known as intracellular adhesion molecules (ICAMs) (Kelly et al., 2007; Weiss and Walcheck, 2008). There are 3 different ICAMs, ICAM-1, ICAM-2, and ICAM-3, which respectively bind to resting epithelium, endothelial cells, and leukocytes (Weiss and Walcheck, 2008; Borregaard, 2010). Specifically, it is the down-regulation of CD62L along with CD18, which contribute to the observed neutrophilia in the blood following a stressor or an increase in glucocorticoids (Tempelman et al., 2002). Once in the tissue, neutrophils are activated by the binding of their TLR to bacterial proteins, which initiates a signaling cascade resulting in the neutrophil dispelling its granules at the infected site/cell. Neutrophils release IL-1β in response to the pathogens they encounter, triggering the acquired immune response (Carroll and Forsberg, 2007).

After phagocytosis of a pathogen, neutrophils act to kill invaders through their generation of reactive oxidative species. This process takes place inside the phagosome of the neutrophil and is commonly known as the respiratory burst, as it involves a large increase in oxygen consumption by the cell. The reduction of NADPH and the oxidation of oxygen by the enzyme complex, NADPH oxidase, results in the production of the superoxide anion ($O_2^-$). The NADPH oxidase is an enzyme complex that consists of both membrane bound and cytosolic components. The membrane bound cytochrome $b_{558}$, is a herterodimer composed of 2 polypeptide subunits, p22-*phox* (the α-subunit) and gp91-*phox* (the β-subunit), along with a flavin adenine dinucleotide (FAD) (McPhail et al., 1992; Sheppard et al., 2005). Together they act to transfer electrons from the cytosol across the phagolysomal membrane (Sheppard et al., 2005). Once the cell has been stimulated, a g-protein from specific granules, rap-1A, translocates along with cytochrome $b_{558}$ to the plasma membrane (Sheppard et al., 2005). The cytosolic parts of the oxidase enzyme complex are p40-*phox*, p47-*phox*, and p67-*phox*, and Rac-1 or Rac-2 (McPhail et al., 1992; Babior, 1999; Clark, 1999). Defects in the *phox* genes are commonly found in patients with chronic granulomatous disease (CGD) (McPhail et al., 1992; Clark, 1999). Patients with CGD are more susceptible to infections and subsequently clear infections at a slower rate (Clark, 1999). The $O_2^-$ produced in the respiratory burst will act as a substrate for the production of $H_2O_2$ by the enzyme superoxide dismutase. After the creation of $H_20_2$, in the respiratory burst, additional reactions take place which create additional reactive oxidative species that are highly toxic to bacteria. Myeloperoxidase (MPO) inside the azurophilic granules aids the

reaction of $H_2O_2$ with halides to create hypochlorous acid (HOCl), which is the most effective against bacteria (Witko-Sarsat et al., 2000; Weiss and Walcheck, 2008). During the respiratory burst, reactive oxidative species are formed which have potent bactericidal capabilities. Because of neutrophils' abilities to mount this innate immune defense, they are considered one of the most important cells of the innate immune system. However, just as the various ROS are capable of killing bacteria, they are also implicated in damage to the host if the inflammatory response is not quelled after its destruction of bacteria or pathogens.

**Neonatal Immunity**

The immune system of neonates is naïve and its proper development is essential for their survival. In cattle as in many other mammalian species, calves are born naïve, with low levels of immunoglobulins (Igs) and immune function. This naïve state is known as aggamaglobulinemia (Weaver et al., 2000). Ingestion of colostrum is essential for calves to obtain the immunoglobulins and antibodies by way of passive transfer from their mothers necessary for their survival. Increases in Igs in calf sera can be seen within hours of colostrum consumption (Merriman, 1971). Differences in WBC counts and their differentials are evident between colostrum deprived and colostrum fed calves (LaMotte and Eberhart, 1976). Calves with the lowest IgG concentrations had decreased survival rates (McEwan et al., 1970), higher morbidity rates, along with lower average daily gains (ADG) (Dewell et al., 2006). The greatest percentage of neonatal mortality in calves occurs during the first month of life (Singh et al., 2009), with males having greater mortality rates than females (Patterson et al., 1987; Nix et al., 1998). Preweaning

morbidity and mortality account for reductions in weaning weights (15.9 kg), with one of the major causes of morbidity being respiratory disease (16.5 kg weaning weight reductions) (Wittum et al., 1994; Wittum and Perino, 1995).

Neonates are known to have suppressed abilities to respond to various pathogens. Evidence supporting this is seen in studies which utilized lymphocytes from 1, 2, 3, 7, and 10 d old calves. Cells from newborn calves had decreased blastogenic responses to Con A and phytohemmagglutinin (PHA) when compared to lymphocyte responses of 10 d old calves (Manak, 1986). Furthermore, the addition of cortisol to cell cultures leads to further decreases in lymphocyte responses (Manak, 1986).

Interestingly, neutrophils from neonatal calves were shown to have increased migratory abilities *in vitro* when compared to neutrophils of adult cattle (Zwahlen and Roth, 1990). A study examining neutrophils' ability to produce reactive oxidative species (ROS) in calves from birth through 4 months of age reported neutrophil ROS capacity to increase with calf age (Costa et al., 2008). In contrast, another study examining calf neutrophils through 6 months of age revealed the greatest respiratory burst activity during the first week of life and small decreases through the first 2 months of life (Kampen et al., 2006). The reduced oxidative burst activity shown in neutrophils of young calves, can partially be attributed to their reduced myeloperoxidase activity (Lee and Roth, 1992).

As the fetus develops, parts of the immune system are activated and able to respond to stimuli. The timing of development of organs such as the lymph nodes, spleen, liver, and thymus represent possible critical windows where disturbances are able

to alter the functioning of organs and cells controlling the immune response. Cells producing IgM were observed as early as day 59 of gestation, while IgG containing cells weren't observed until gestation day 145 (Schultz et al., 1973). Stressful stimuli during days 60 through 140 or 150 of gestation would likely be able to alter the production of these and other immune cell populations in the fetus. Changes in the fetus's ability to mount an immune response can have negative effects on its growth, health, and survival, both *in utero* and later in its life. Since gestation length in cattle is similar to that of humans, cattle may possibly serve as an animal model when looking at aspects of immune function in neonates throughout development.

**Immune Function and Stress**

Similar to the other systems of the body, stress can have both positive and negative effects on the immune system depending on the duration and type of stressor the individual is enduring (Dhabhar, 2009). Immune function in animals is known to be reduced in response to stress through the inhibition of leukocytes to sites of inflammation, reducing macrophages and their ability to differentiate and recruit other immune cells through their production of cytokines (Boumpas et al., 1993). The numbers of circulating eosinophils and basophils are also typically decreased due to exposure to glucocorticoids (Boumpas et al., 1993). A session of brief acute stress in rats resulted in decreased leukocyte populations, likely induced by the increased cortisol which subsequently recovered after the end of the stressor (Dhabhar et al., 1995). Sound stress in male rats resulted in decreased neutrophil ROS and phagocytosis, which was

reversed upon adrenal medullectomy, indicating that epinephrine also plays a role in altering immune function (Brown et al., 2008).

Conversely, numerous studies indicate enhancement of immune function during instances of stress. In cattle, transport for 9 h increased circulating neutrophil numbers, indicative of an inflammatory response (Buckham Sporer et al., 2007). The same study also found altered gene expression of 4 neutrophil genes (Fas; was down-regulated, while l-selectin, MMP-9, and BPI were up-regulated) involved in regulation of the inflammatory response in young beef bulls (Buckham Sporer et al., 2007). Similarly, *in vivo* administration of dexamethasone (DEX); a commonly used glucocorticoid, to cattle resulted in increased migration of polymorphonuclear cells (PMN) from the bone marrow. Despite increased responses of PMN, DEX impaired their phagocytic ability, oxidative metabolism, and cytotoxic capacity to respond to an *in vitro* bacterial challenge (Roth and Kaeberle, 1981). Additionally, transport of dairy cattle showed a decrease in apoptotic neutrophils until 1 d after unloading the cattle (Yagi et al., 2004). An increase in circulating neutrophils at 2 h after unloading, accompanied this decrease in apoptosis, demonstrating neutrophillia to be inversely related to apoptosis (Yagi et al., 2004). Increased NKCs were observed after 2 h of transport, along with an increased neutrophil to lymphocyte ratio (Ishizaki and Kariya, 2010).

**Weaning as a Stressor**

Weaning is a stressful but necessary process in the life of an animal. It involves the forced severing of the bond between mother and offspring. Additionally, it is the time when an animal begins to truly be on its own. Behavioral and physiological

changes occur in both the mother and offspring in response to weaning (Malinowski et al., 1990; Pollard et al., 1993). Studies have shown increased cortisol concentrations (Crookshank et al., 1979; Malinowski et al., 1990; Kojima et al., 2008) and weight loss accompanying decreased ADG (Pollard et al., 1993) in response to weaning. Immune function has also been shown to be negatively affected by weaning stressors. Weaning resulted in increased neutrophils, along with simultaneous decreases in lymphocytes (Blanco et al., 2009; Lynch et al., 2010; Kim et al., 2011; O'Loughlin et al., 2012).

In production animals, the severing of the bond at weaning is often not the only stressor the animal will endure. Many animals are weaned just prior to transport, comingling and sale; all of which are additional known stressors. Together these may leave animals more susceptible to diseases (Crookshank et al., 1979). Proper management decisions can help to reduce the impact of these stressors and improve animal health; ultimately increasing animal welfare and decreasing economic losses.

**Temperament**

Temperament has been defined as a behavioral measurement of the fear response to humans or novel environments (Burrow, 1988). The temperament of cattle is known to be a repeatable measurement (coefficient of repeatability = 0.48) (Kilgour et al., 2006) of a moderately heritable trait that influences birth weights, growth, average daily gains (ADG) (Voisinet et al., 1997b; Fell et al., 1999), bruising, meat quality (Voisinet et al., 1997a) and response to stressors (Burrow and Dillon, 1997). Measurements of temperament in cattle are comparable to measurements of high and low anxiety in rats. It is also similar to humans, whereby one person can be calm and mellow when

experiencing daily stressors, while another person may overreact to daily perturbations and exhibit a greater a stress response.

Assigning cattle a temperament score, entails averaging two common methods of temperament assessment, their exit velocity (EV) and their pen score (PS). The exit velocity (m/s) is calculated by recording the length of time it takes cattle to exit a working chute and traverse a set distance of 1.83 m (Burrow, 1988; Curley et al., 2008). The pen score is assessed by an unbiased observer based on the calf's behavior and aggressiveness during interactions with other calves and toward the observer while in the pen (Hammond et al., 1996; Burdick et al., 2010; Burdick et al., 2011a; Burdick et al., 2011b). Animals are scored on a basis or 1 to 5, with 1 = calm and 5 = aggressive.

Stress is known to result in temperamental cattle having greater concentrations of cortisol and catecholamines than calm cattle (Curley et al., 2008; Burdick et al., 2010). Additionally, temperamental cattle displayed greater peak ACTH concentrations following administration of a VP bolus (Curley et al., 2010).

The temperament of cattle has recently been implicated in differences in innate immune function. Researchers found temperamental bulls to have increased neutrophil to monocyte ratios compared to calm cattle after transport (Hulbert et al., 2011). Increased neutrophil numbers are suggested to be indicative of a stress response. Temperamental cattle had greater resting rectal temperatures prior to transportation than intermediate or calm cattle (Burdick et al., 2010).

**Transport**

Transport has been shown to be a stressor across animal species with negative impacts on their welfare. Reductions in body weight (shrink) (Kannan et al., 2000; Earley et al., 2010) and meat quality (due to increases in meat pH and dark cutters) (Jones and Tong, 1989), changes in immune (Ainsworth et al., 1991; McGlone et al., 1993; Gupta et al., 2007; Ishizaki and Kariya, 2010) and metabolic functions (Buckham Sporer et al., 2008; Earley et al., 2010) (glucose and lactate concentrations) (Iversen et al., 1998), increased bruising, injuries (Minka and Ayo, 2008), susceptibility to diseases and mortality (Voslarova et al., 2006) are just some of the ramifications of transportation (Tarrant et al., 1992; Speer et al., 2001; Cernicchiaro et al., 2012). Along with the consequences to animal welfare, transportation leads to economic losses (Speer et al., 2001).

Transportation of animals elicits the release of cortisol through its activation of the HPAA (Lay et al., 1996; Duvaux-Ponter et al., 2003). This results in increased serum (McGlone et al., 1993; Iversen et al., 1998; Fazio et al., 2008) and salivary (Fell and Shutt, 1986) cortisol concentrations in stressed animals. Simultaneously, elevated rectal temperatures have been observed as an indicator of a stress response due to transport (Minka and Ayo, 2008; Burdick et al., 2010; Burdick et al., 2011b; Maeda et al., 2011).

Transportation stress also decreases immune functions in animals. Holstein calves that underwent 4 h of transport had decreased neutrophil counts and blastogenic

responses to mitogens at 0 and 4 h after transport; with normal concentrations and activity returning by 4 d post transport (Murata et al., 1987).

In contrast, transport has also been shown to increase immune function. Transport appears to positively affect innate immune parameters such as neutrophils. For instance, transport for 4 h increased circulating neutrophil concentrations, the neutrophil:lymphocyte ratio and NK cell cytotoxicity in pigs (McGlone et al., 1993). In cattle transport for 9 h increased neutrophil and WBC counts (Buckham Sporer et al., 2007; Buckham Sporer et al., 2008).

In cattle production, producers sell pregnant cows. This means the cattle will be transported various distances depending upon where the buyer's property is. In certain environments, producers have one pasture for spring/summer and another pasture in a different location for winter/fall. This would require additional transportation of the herd. Each time producers move their animals, they induce stress upon them. Additionally after transport, cattle are exposed to a novel or different environment, which places additional stress on the animals. This leaves them more susceptible to infections.

Bovine respiratory disease (BRD) is considered one of the most common threats to cattle, as it results in increased incidents of mortality and morbidity (Snowder et al., 2006; Duff and Galyean, 2007). This is particularly true for calves from birth through age of weaning. Susceptibility to BRD is impacted by several factors; a few of which include transportation, commingling, and stress on the animals (Snowder et al., 2006; Duff and Galyean, 2007).

*Transport During Gestation*

When pregnant animals endure transportation, their offspring are subjected to adverse conditions. This has potential to alter the offspring in several ways. Despite ongoing studies on transportation stress and prenatal stress, very few experiments exist that incorporate both of these aspects into their experimental designs. This is particularly interesting as pregnant production animals may experience transportation. Moreover, a prenatal stressor such as transportation represents a typical managerial stressor rather than a pharmacological manipulation of the HPAA. Understanding the ramifications transportation has on gestating production animals and their offspring would allow for the creation of handling procedures and management tactics that would benefit the offspring and the industry.

Prenatally transported ewes gave birth to lambs that spent more time exploring novel environments, more time close to novel stimuli, and spent less time jumping when isolated than unstressed controls (Roussel-Huchette et al., 2008). These behaviors indicate prenatal transportation made lambs less fearful than controls, most likely through alterations in their HPAA while the lambs were *in utero* (Roussel-Huchette et al., 2008). Recent work in our laboratory demonstrated differences in calf temperament in response to repeated prenatal transportation stress. Prenatally stressed calves were more temperamental compared to controls (Littlejohn et al., 2013). Less fearful and more temperamental animals may pose a risk to themselves and to their handlers.

Similarly, repeated prenatal transportation of Brahman cows was shown to alter the physiological response to stressors in transported (TRANS) calves (Lay et al.,

1997b). Calves that endured repeated prenatal transportation stress displayed increased adrenal activity and a decrease in their ability to clear cortisol after a challenge (Lay et al., 1997b).

Repeated transportation in isolation during gestation was also studied in goats. However, unlike the ewes and cows, goats did not habituate to the stressor (Duvaux-Ponter et al., 2003). This may have been related to the fact that this study involved transport and isolation, each of which by themselves causes a stress response in goats and sheep (Duvaux-Ponter et al., 2003). The impact transportation stress has on the offspring of transported animals needs further examination as studies have yet to clarify the effects it has on offspring's immune system.

Since temperament is heritable and has been shown to affect an animal's innate immune response, an animal's ability to mount an immune response is likely heritable as well. The effect of temperament on the stress response and the alterations it causes to offspring of gestational stress are likely exhibited through changes in gene expression. Recent research involving epigenetics has shown alterations in an animal's gene expression through methylations and/or histone acetylations of DNA are capable of inducing changes in tissue functioning and differentiation (Zakharova, 2009). These changes have been shown to be heritable by subsequent generations (Zakharova, 2009). Taking this into consideration, one can easily see there are many levels of complexity involved in how stress affects an animal and its offspring. Therefore, the modifications temperament and stress have on offspring immune function necessitate further examination. Stress plays a pivotal role in the developing animal and has vast effects on

an animal's body and its systems. More research in the area of prenatal stress needs to be done with respect to its impacts on the developing offspring and their response to being transported while *in utero*.

Objectives:

1. To determine the physiological effects in gestating Brahman cattle resulting from repeated transport.

2. Examine the effects prenatal transport has on immune and stress systems of the offspring prenatally stressed *in utero*.

# CHAPTER II

# PHYSIOLOGICAL RESPONSES TO REPEATED TRANSPORTATION OF GESTATING BRAHMAN COWS

## Introduction

Over the course of their life, production animals are subject to transportation. Transportation is considered a stressor for animals and results in economic losses for the production industry each year (Speer et al., 2001). How an animal reacts during handling and transport is related to a variety of factors including their temperament, handling procedures, ambient temperature, season of the year, temperature in the trailer, stocking density (Tarrant et al., 1992), amount of commingling (Arthington et al., 2003) and the distance of the journey (Fazio et al., 2008). Understanding aspects affecting animals during transportation can help improve animal welfare, management practices and handling techniques; ultimately, reducing economic losses for the production industry.

Upon encounter with a stressor, the hypothalamic-pituitary-adrenal axis (HPAA) of the animal is activated, resulting in the release of cortisol from the adrenal cortex and catecholamines from the adrenal medulla (Sapolsky et al., 2000; Chrousos, 2009). Cortisol and catecholamines are then able to affect other systems in the animal's body, causing changes in metabolic, immune, and reproductive functions (Sapolsky et al., 2000; Chrousos, 2009).

The temperament of an animal is defined as a behavioral measurement of the fear response and is a commonly used assessment in cattle (Burrow, 1988). Temperament is known to play a role in an animal's response to stress with temperamental animals displaying greater cortisol and catecholamine concentrations after encountering a stressor when compared to calm animals (Stahringer et al., 1990; Curley et al., 2006; Curley et al., 2008; Burdick et al., 2010). Additionally, temperamental animals are known to have poorer meat quality, lower ADG and may be problematic if handlers are not properly trained to work with these animals (Voisinet et al., 1997a; Voisinet et al., 1997b).

Previous work from our group demonstrated that pregnant cattle are affected by transportation stress, evidenced by their changes in body weight, percent shrink and circulating cortisol concentration (Lay et al., 1996). Coupling this concept with knowledge that temperament affects an animal's response to a stressor, we investigated if the temperament of pregnant Brahman cattle affected their responses to transportation. Thus the objective of this experiment was to observe the physiological influence repeated transportation has on gestating Brahman cows.

## Materials and Methods

### *Animals*

The Brahman cows in the Overton Research Center's herd were artificially inseminated for this experiment. Pregnancy was confirmed by palpation per rectum and compared with records of insemination dates. Cows remained untouched prior to transport. Brahman cows (N= 48) for this experiment were matched for temperament,

age and parity. Each cow was classified into one of three temperament groups: 1) calm (N= 10), 2) intermediate (N= 28), 3) temperamental (N= 10) based on their temperaments determined at weaning and again as adult cows (Stahringer et al., 1990).

*Experimental Design*

The cows were transported by trailer for a duration period of 2 h on each of days (d) 60, 80, 100, 120, and 140 ± 5 d of gestation. At least 24 h prior to each transport event, vaginal indwelling devices (Burdick et al., 2012) were inserted into the cows and vaginal temperatures were recorded prior, during, and through 30 min post cessation of transport. On each transport day, cows had their weight recorded and blood samples were collected before and after transport. The percent shrink of each TRANS animal was calculated for each transport day. Weather data for each date of transport was obtained from the Texas A&M Agrilife weather station in Overton, TX.

Blood samples were collected via puncture of the tail vein into 15 mL red top (no additives) tubes. Collected blood was refrigerated overnight at 4º C and centrifuged at 1700 G (~3000 rpm) for 20-25 min at 6-7º C the following day to allow for maximum yield. The serum was pipetted off and stored in aliquots at -80º C until processing.

*Cortisol RIA*

Cortisol concentrations were determined from duplicate samples using a single antibody RIA procedure (Willard et al., 1995; Curley et al., 2010) and utilized: rabbit anticortisol antiserum (Pantex, Div. of Bio-Analysis Inc., Santa Monica, CA, Cat. #P44) diluted 1:2500; standards made by serial dilution (8000 pg/100 µL to 3.9 pg/100µL) of 4-pregnen-11β,17,21-triol-3,20-dione (Steraloids Inc., Newport, RI, Cat. #Q3880-000);

and radio-labeled cortisol: 3H-Hydrocortisone (1,2-3H, NEN, Boston, MA, Cat. #NET-185). Counts per minute (cpm) were obtained from a liquid scintillation spectrophotometric beta-counter (Beckman Coulter LS 6500) and unknown cortisol concentrations were calculated using Assay Zap software (Biosoft, Cambridge, UK). Cortisol antiserum cross-reactivity was: corticosterone, 60%; deoxycorticosterone, 48%; progesterone, 0.01%; and estradiol, 0.01%, (determined by Pantex). Interassay and intraassay CV were 11.89% and 13.32%, respectively. The average total cpm per tube was 10922 counts. The average max binding and non-specific binding was 34.6% and 9.78%, respectively. Data from this assay are presented in ng/mL.

*Glucose Assay*

The Wako Autokit for Glucose (Code-No. 439-90901) was used to determine the glucose concentrations of the samples. The samples were processed in duplicate. Tubes were set up for standards, samples, and a blank prior to the start of experiment, with 0.02 mL of standard and 0.02 mL of sample pipetted into their appropriate tubes. Then 3.0 mL of working solution was pipetted into the standard, sample and blank tubes. The tubes were vortexed for several seconds to mix them, followed by 5 min of incubation. The absorbance was measured at 505 nm on a spectrophotometer.

*NEFA Assay*

The enzymatic HR Series NEFA-HR (2) assay (Wako Diagnostics, Richmond, VA) was modified to fit a 96-well format and used to determine NEFA concentrations. Briefly, 200 µL of the Color Reagent A was prepared. Then in a 96-well plate, Color Reagent A was added to 5 µL of serum or standards made by dilution of the stock solution with distilled water. The plate incubated for 5 min at 37$^{o}$C and a spectrophotometer was used to quantify the absorbance at 550 nm. Next, 100 µL of Color Reagent B was made and added to all the wells on a 96-well plate. The plate then incubated for an additional 5 min and absorbance was read for a second time using a plate reader at 550 nm. The plate reader for this assay required an incubating and timing feature. This ensured accurate absorbance readings of the samples immediately following the 5 min incubation. The first reading was multiplied by a factor of 0.67 to account for changes in volume and was then subtracted from the second reading to obtain the final absorbance. All calculations (i.e., standard curve, samples concentrations) are based on the final absorbance values. NEFA concentrations were determined by comparing unknown samples to a standard curve of known NEFA concentrations. The minimum detectable concentration for this assay is 0.0014 mmol/L and the intra- and inter-assay coefficients of variation are 7.16% and 11.26%, respectively. Data are presented as the concentration in mmol/L.

*Statistical Analysis*

All statistical analysis was carried out using SAS software (SAS, 2011). The vaginal temperatures and serum concentrations of cortisol, glucose, and NEFA were

analyzed using GLM procedures specific for repeated measures. The area under the curve for vaginal temperatures was calculated using the trapezoidal rule and analyzed with GLM procedures. The area under the curve for the vaginal temperatures was determined utilizing the following equation:

AUC VT = $\Sigma$ (((VT$_n$ + VT$_n$ + 1)/2) × d); where VT is the vaginal temperature at a given time point and d is the time in days between the two vaginal temperatures.

**Results**

The physiological effects of repeated transportation of pregnant Brahman cows examined in this experiment included vaginal temperatures, serum cortisol and glucose concentrations, plasma NEFA concentrations, and the percentage of weight lost (shrink). Transportation was found to be a stressor in pregnant Brahman cows.

*Vaginal Temperatures*

When examining the vaginal temperatures (VT) prior to transportation (Figure 1), the mature temperament score affected the VTs ($P < 0.03$), whereby calm cows exhibited greater basal VTs than the temperamental cows. Time also significantly affected ($P < 0.0001$) the basal temperatures, with slight decreases in VT on the day of each subsequent transport event. All the basal temperatures on each transport day regardless of temperament were between 37 and 38.5 ºC. Additionally, there was a tendency for an interaction between the mature temperament score and time, ($P = 0.0877$). All cows, regardless of temperament exhibited an increase in VTs during each transport event (Figure 2). The change in VTs was affected by mature temperament score ($P < 0.02$), as the greatest change from basal temperatures was seen in the

temperamental cows during each instance of transportation. There were no interactions ($P > 0.1$) affecting the changes in VTs. Additionally, the mature temperament score had a tendency ($P < 0.09$) to affect the peak VT during transport (Figure 3), with the temperamental animals having the numerically greatest peak VTs. The calm cows' peak VTs were repeatedly observed to be lower than the temperamental cows' peak VTs. However, time significantly ($P < 0.01$), affected the peak VT during transport with all the animals regardless of temperament having the greatest peak VTs on the first instance of transport, d 60. There was no mature temperament score by time interaction ($P > 0.3$) detected for the peak VT during transport. The area under the curve (AUC) was calculated for the VTs to examine the effect of mature temperament score on VTs over time. A significant effect of time ($P < 0.01$; Figure 4) was seen for the AUC VTs, as all cows had increases in VT during all transportation events. However, there was no effect of the mature temperament score ($P > 0.2$), nor was there a mature temperament score by time interaction ($P > 0.5$) on the AUC for VTs.

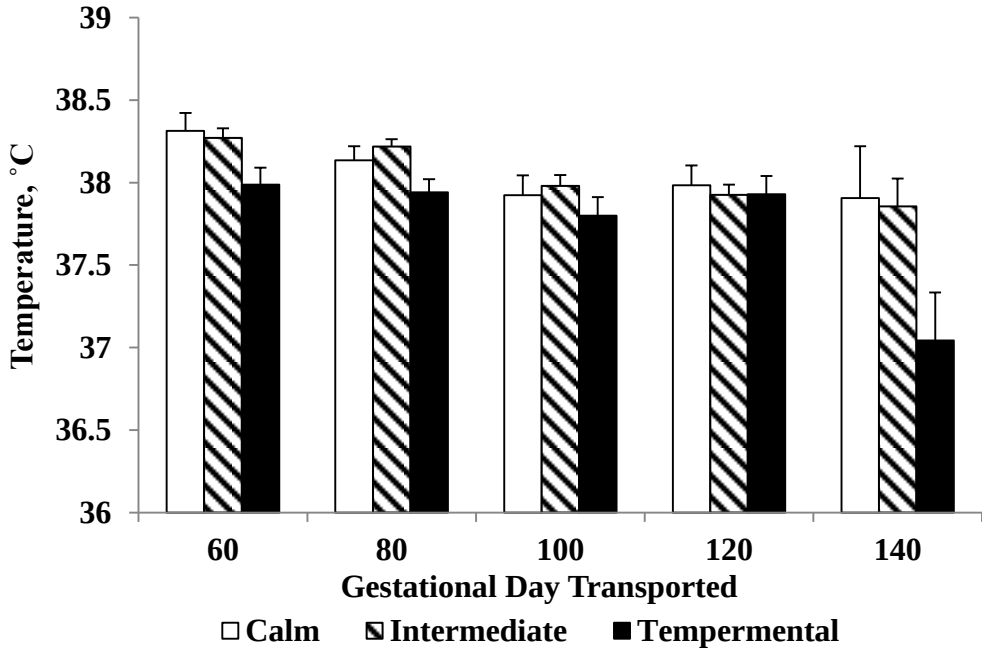


**Figure 1.** The effect of the mature temperament score on pre-transport basal temperature. The greatest basal vaginal temperatures (VTs) were observed in the calm cows, ($P < 0.03$). The basal VTs of all cows decreased at each subsequent day of transport.

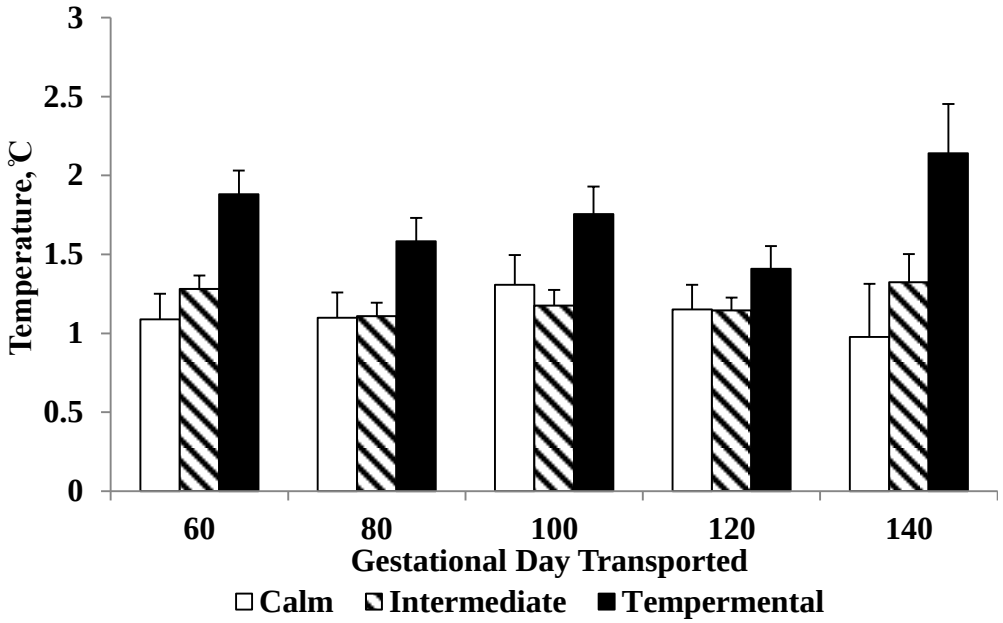


**Figure 2.** Effect of mature temperament score on differences in peak and basal transport temperatures. During each transport event, the temperamental cows displayed the greatest change in VT from pre-transport basal temperatures.

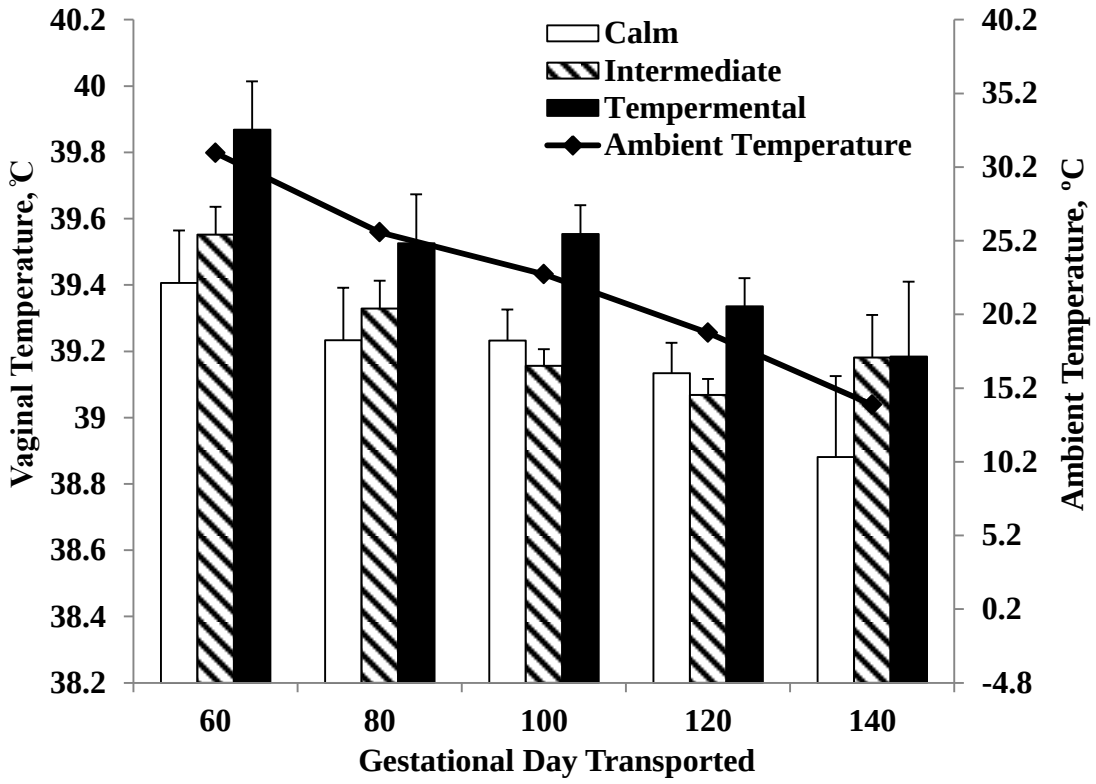


**Figure 3.** Effect of mature temperament on peak transport vaginal temperature. The temperamental cows consistently displayed the largest peak in vaginal temperatures (VTs) over all transport events, ($P = 0.0851$). The peak VTs in all cows decreased with subsequent transport events, ($P < 0.0001$). This agreed with the decrease in average ambient temperature observed over the course of the study.

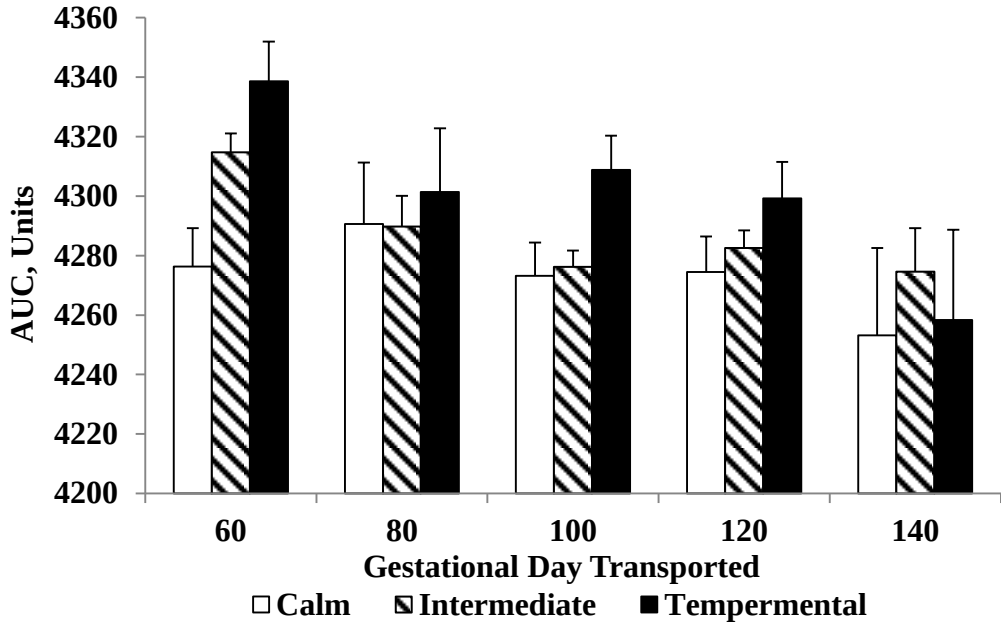


**Figure 4.** Effect of mature temperament on AUC during transport. All cows exhibited decreased changes in vaginal temperatures (VTs), with additional transport events, ($P = 0.002$).

*Cortisol*

The mature temperament score affected the cortisol response of pregnant Brahman cows (Figure 5; $P < 0.01$), with temperamental animals consistently displaying greater cortisol concentrations pre-transport when compared to both calm and intermediate cows. Time also affected the serum cortisol concentrations pre-transport ($P < 0.02$), as they differed over the span of transport events. There was no time by temperament interaction ($P > 0.4$) observed for serum cortisol concentrations pre-transport. Examining cortisol differences between post and pre transport (Figure 6), there were no effects of time ($P > 0.5$), or temperament ($P > 0.9$) alone, however, there was a tendency for a temperament by time interaction ($P = 0.102$). All cows regardless of temperament displayed cortisol differences during each instance of transport. The differences on d 140 were less than the differences on d 60, the first instance of transport.

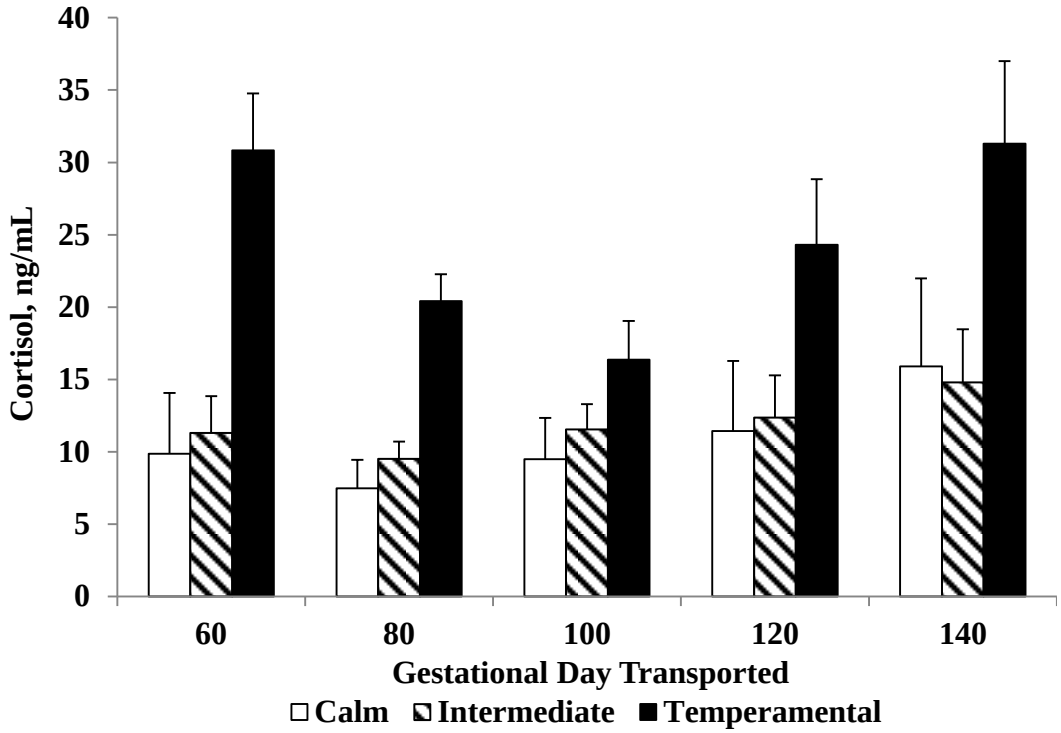


**Figure 5.** Effect of mature temperament on pre-transport cortisol concentration. Temperamental cows consistently displayed the greatest cortisol concentrations, ($P = 0.0002$). All cows elicited variations in cortisol concentrations over time, ($P = 0.0134$).

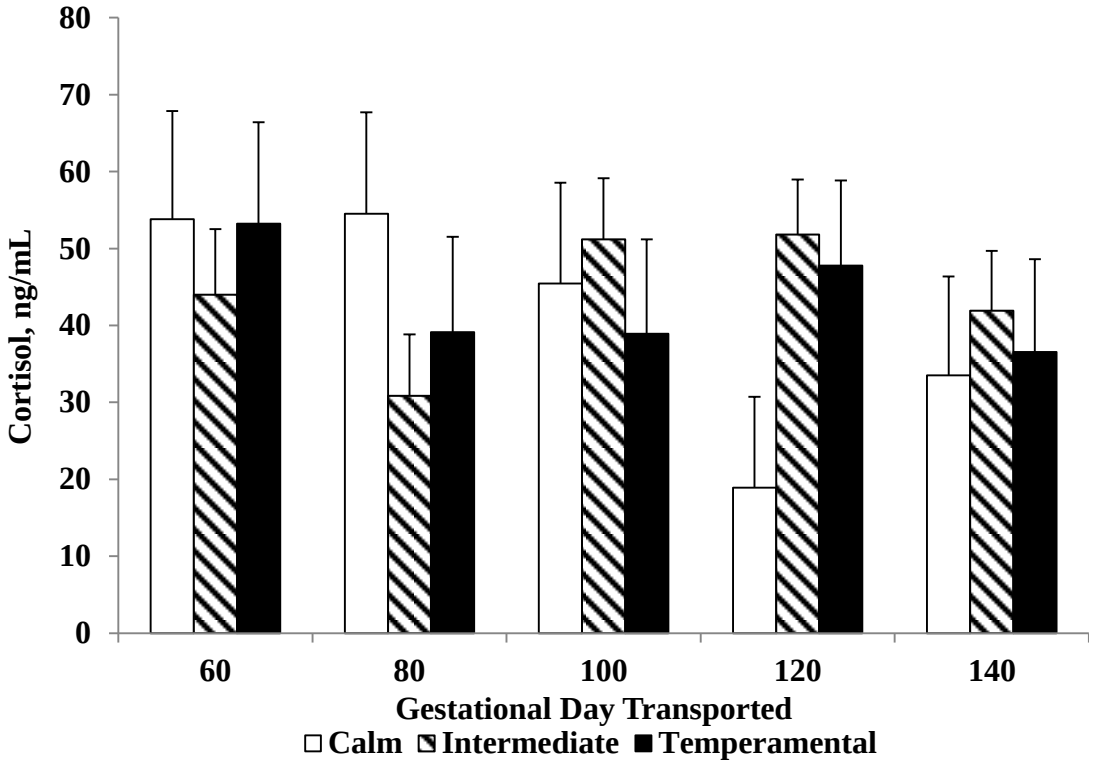


**Figure 6.** Effect of mature temperament on differences in cortisol concentrations between pre and post transport. All cows, regardless of temperament ($P > 0.1$) demonstrated changes in cortisol concentrations during each instance of transport.

*Glucose*

Similar to our results for cortisol, serum glucose concentrations pre-transport (Figure 7) were greater ($P < 0.03$) in temperamental cows than in calm cows for all transport events. The serum glucose concentrations pre-transport were also affected by time ($P < 0.01$). There was a tendency for a temperament by time interaction ($P = 0.1026$). When calculating the differences in pre and post transport serum glucose concentrations (Figure 8), temperament ($P > 0.1$) had no effect on the pre and post transport glucose differences. However, there was a significant effect of time ($P < 0.001$) and a temperament by time ($P < 0.02$) interaction. All cows had decreased differences in glucose concentrations with each subsequent transport event. Interestingly, while both the calm and intermediate cows steadily displayed less change in glucose concentrations with each subsequent transport event, the temperamental cows showed the slowest propensity to exhibit less change in glucose concentrations.

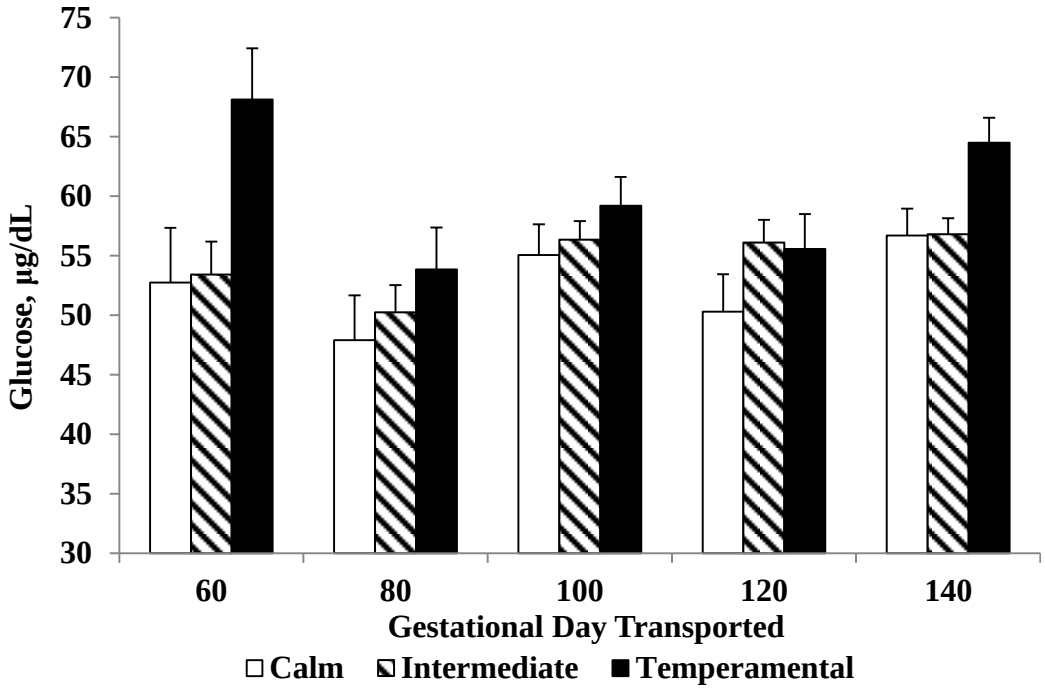


**Figure 7.** Effect of temperament on pre-transport glucose concentration. The temperamental cows consistently have the greatest pre-transport glucose concentrations, ($P$ = 0.0230).

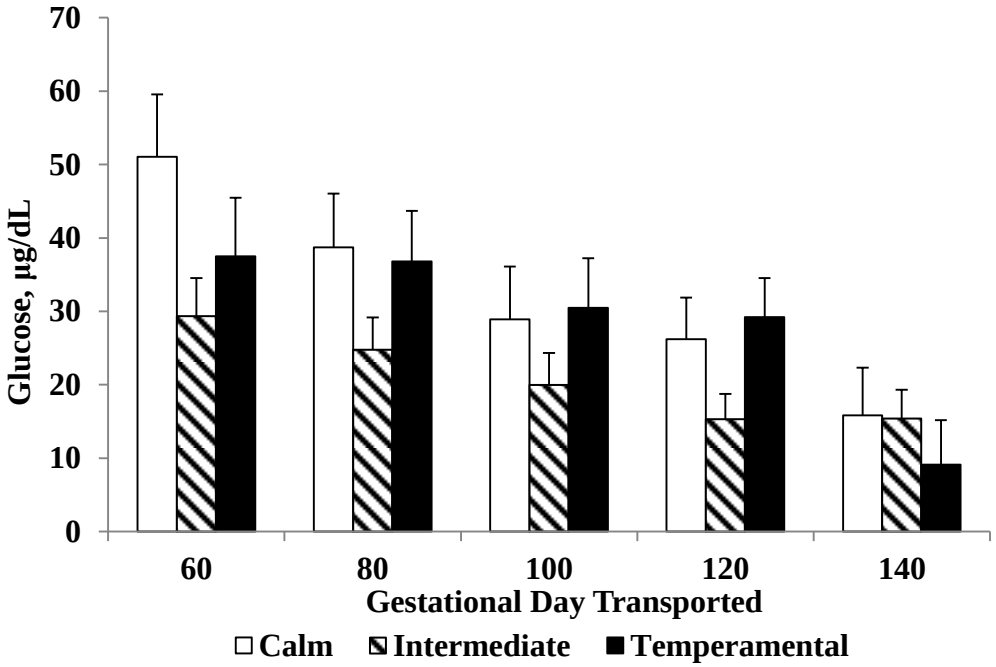


**Figure 8.** The effect of temperament on the differences between pre and post transport glucose concentrations. By d 140 of transport, all cows displayed less change in glucose concentrations in response to transportation, ($P < 0.0001$).

*NEFA Concentrations*

Investigations of serum NEFA concentrations resulted in no effect of temperament ($P > 0.3$) or time ($P > 0.8$) on post transport NEFA concentrations (Figure 9). No interaction was observed for post transport NEFA concentrations. Over the course of transport events, the NEFA concentrations displayed few differences amongst temperament groups. Observations of the differences in NEFA concentrations between post and pre-transport values (Figure 10) yielded few differences in NEFA concentrations. The differences in NEFAs due to transport were affected by temperament ($P = 0.0099$), with temperamental cows having the least change in NEFA concentrations compared to calm and intermediate cows. Time ($P = 0.0002$) also affected the change in NEFA concentrations, as all cows exhibited different changes in NEFA concentrations at each transport event. There was no interaction ($P > 0.1$) observed.

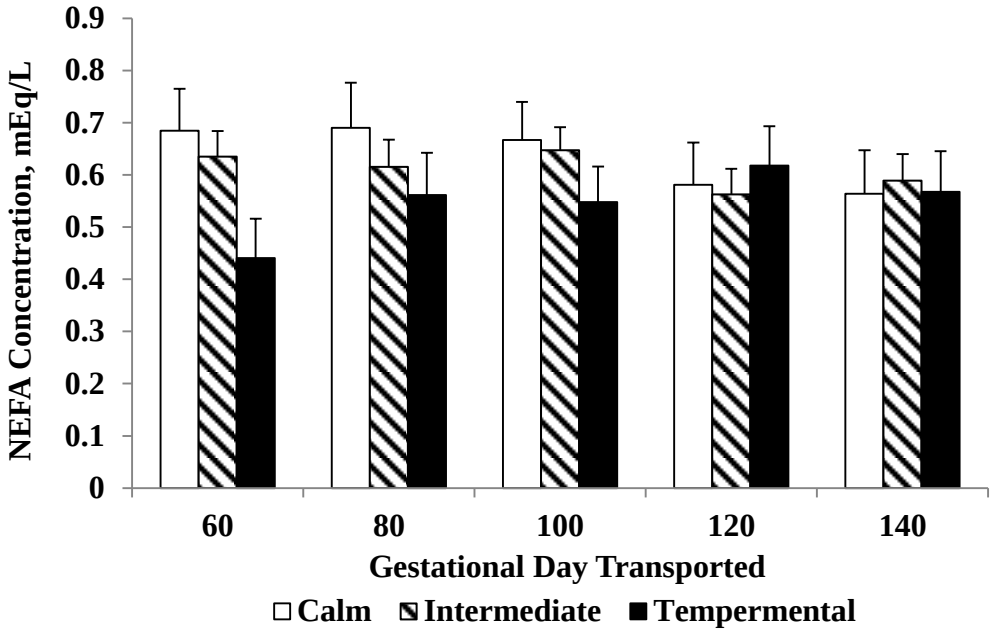


**Figure 9.** Effect of temperament of NEFA concentration post transport. The NEFA concentrations remained relatively stable after each instance of transport, ($P > 0.8$).

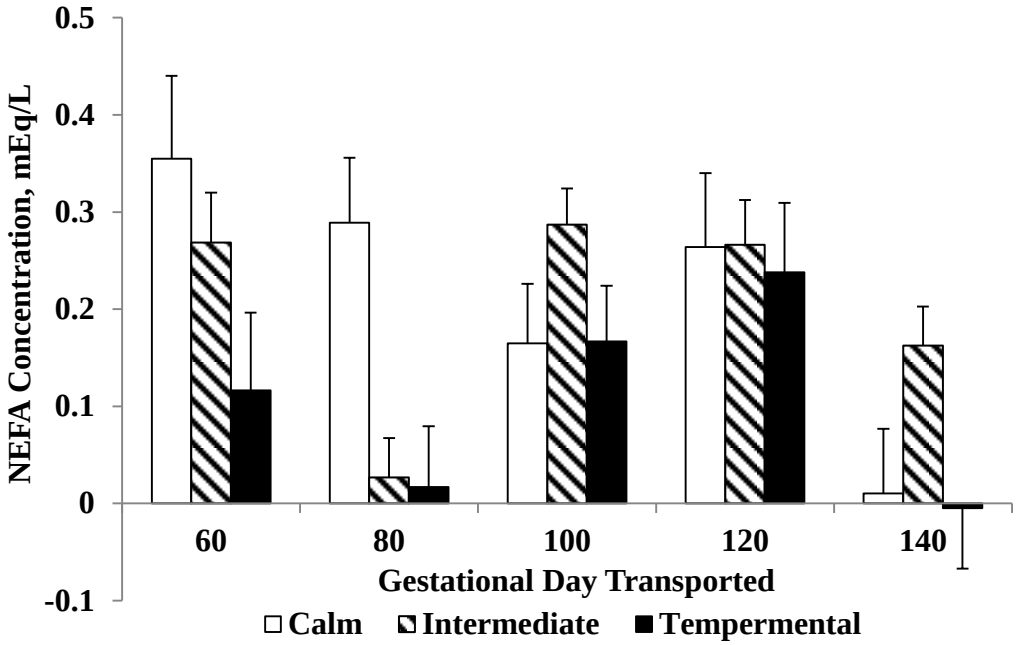


**Figure 10.** Effect of temperament on the differences in NEFA concentrations between pre and post transport. All of the cows exhibited different changes in NEFA concentrations over time, ($P$ = 0.0002).

*Shrink*

The percent shrink was calculated from cow body weights taken before and after each transport event. All cows exhibited shrink during each transport event (Figure 11), with the greatest shrink occurring on d 60, the first instance of transport. There were no effects of temperament ($P = 0.471$), nor were there interactions of temperament with time ($P = 0.7375$). However, time ($P = 0.0008$) affected the percent shrink, as all cows had decreased percent shrink at each additional transport event.

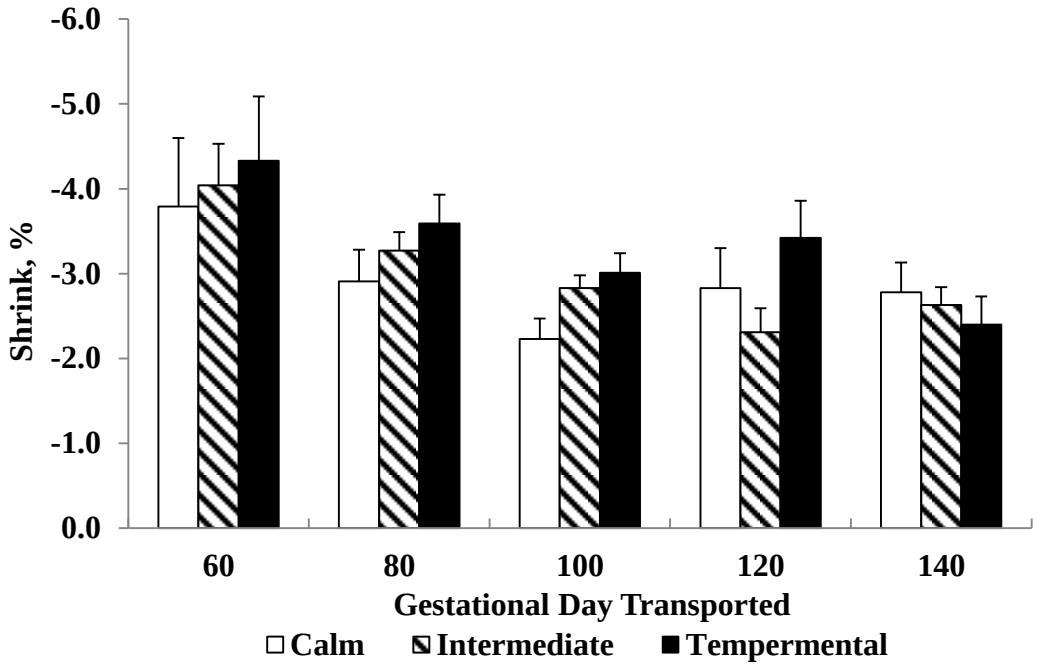


**Figure 11.** The effect of temperament on percent shrink due to transport. The shrink was greatest on the d 60 and decreased with additional transport events, through d 140, ($P = 0.0008$).

**Discussion**

Transport is an inescapable part of cattle management as cattle often undergo transportation. Therefore, it is necessary to understand how such a stressor may affect them. With the array of physiological measurements taken, our data determined transportation to be a stressor for pregnant cows, and that temperament does influence their stress responses. The greater cortisol concentrations exhibited by the temperamental cows pre-transport demonstrates their innate response to handling and entering a chute is greater compared to calm and intermediate animals. This result is consistent with studies showing that handling and loading of animals onto a trailer initiates the stress response and increases release of cortisol (Trunkfield and Broom, 1990; Minka and Ayo, 2008). Our experiment reaffirms transportation as a stressor in pregnant cattle (Lay et al., 1996) and agrees with previous findings that cortisol responses of cattle to transportation vary with the animal's temperament (Stahringer et al., 1990; Curley et al., 2006; Curley et al., 2008).

Recent work in dairy cattle has validated agreement between rectal and VT, providing support for the comparison of these physiological measures (Suthar et al., 2013). We observed increases in VT in pregnant cows indicative of a stress response due to transportation. These results are expected as other studies have shown transportation to be stressful evidenced by increased rectal temperatures in bulls (Burdick et al., 2010; Burdick et al., 2011b). However, contrary to our results in pregnant cows and those on rectal temperature in bulls (Burdick et al., 2010), Burdick et al.'s (2011b) study did not observe an effect of temperament on the changes in rectal

temperatures. This may have been due to the fact that their bulls were allowed to rest on the trailer for 2 h prior to transport. It is believed that the initial handling and loading of animals onto a trailer may be the most stressful aspects of transportation (Trunkfield and Broom, 1990; Minka and Ayo, 2008). The decrease in peak VTs observed over the course of the study in all cows represents their habituation to the stressor. However, some of the reductions in VTs may partially be attributed to decreased ambient temperatures that occurred over the course of the experiment.

The pregnant cows in our study underwent shrink during each instance of transport, proving transport is stressful. They also validated earlier results, that repeated transportation will lead to habituation to the stressor and less shrink during subsequent transports (Lay et al., 1996). Where other studies observed increased NEFA concentrations resulting from transportation (Duvaux-Ponter et al., 2003; Earley et al., 2010), the NEFA concentrations in our cows remained relatively stable. This could have been due to the longer duration of transport utilized in Earley et al.'s (2010) study and the isolation from conspecifics in the goats used by Duvaux-Ponter (2003).

Together, our results demonstrate cattle are able to habituate to a stressor such as transportation over time. These findings are in agreement with other studies showing animals' abilities to habituate to a stressor after repeated exposure to the same stimuli (Lambooy and Hulsegge, 1988; Roussel et al., 2006). Previously, an investigation involving the transport of pregnant heifers either penned or loose found that pregnant heifers displayed less increase in glucose concentrations when an additional transport event was experienced (Lambooy and Hulsegge, 1988). The increase in glucose

concentrations that result from the stress of transportation is likely due to the mobilization of glycogen from muscle stores the initiation of gluconeogenesis. Similar results were observed in sheep that experienced repeated isolation or transportation while pregnant. Over time, both groups of animals were able to habituate to the stressor, as evidenced by their decreased cortisol responses (Roussel et al., 2006). When pregnant goats were repeatedly transported they failed to habituate to the stressor (Duvaux-Ponter et al., 2003). This may have been due to their isolation from conspecifics while they were transported, as isolation is an additional known stressor for sheep and goats (Duvaux-Ponter et al., 2003). However, our work shows it is the temperamental cattle which display less ability to habituate than do calm and intermediate cattle. This supports suggestions for producers to selectively breed cattle with intermediate and calm temperaments over cattle that are temperamental.

The stress experienced by the cattle in our study may ultimately have consequences on their growing and developing calves while they are *in utero*, as increased maternal cortisol concentrations have been implicated as a factor mediating effects on the offspring (Barbazanges et al., 1996; Matthews, 2002). Investigations involving both physiological and behavioral measurements of the calves should be undertaken in order to quantify how transport stress females experience during gestation effects their calves. Examination of the hormonal, behavioral, growth and immune parameters of neonatal calves born to cows transported at several key times during pregnancy will provide insights into the genetic and/or epigenetic effects of prenatal stress on postnatal health and performance.

# CHAPTER III

# PRENATAL STRESS ALTERS ENDOCRINE AND IMMUNE INDICES IN NEONATAL AND GROWING BRAHMAN CALVES

## Introduction

Prenatal stress is known to adversely affect the developing fetus while it is *in utero* (Barker, 2004). Alterations to various physiological systems in the offspring have been repeatedly observed and include changes in metabolic (Vallee et al., 1996) and reproductive functions (Herrenkohl, 1979; Kapoor and Matthews, 2005), behavioral modifications (Clarke and Schneider, 1993), and alterations in immune function (Klein and Rager, 1995; Kay et al., 1998; Coe et al., 2002; Götz et al., 2007; Hodyl et al., 2007). Studies on the effects of stress on immune function often include the use of synthetic hormones such as dexamethasone (DEX), betamethasone (BET) or pharmacological doses as the stressor. However, in livestock production it may be more pertinent to examine methods of prenatal stress which are more likely to be experienced by the animals.

Shortly after the time of birth, animals are known to have low immune function leaving them more susceptible to disease (Weaver et al., 2000). This is particularly true regarding cattle and other ungulates. In fact, the majority of calf mortality is known to occur during the first month of life (Singh et al., 2009). Calf mortality and morbidity are due not only to diseases but are increased in incidences of failure of passive transfer of

immunoglobulins (Igs) from the cow to the calf (McEwan et al., 1970). Failure of passive transfer in calves has been characterized as having a serum IgG concentration of less than 800 ng/ml (Wittum and Perino, 1995; Dewell et al., 2006). Interestingly, male calves as with most mammals display higher mortality rates than female calves (Patterson et al., 1987; Nix et al., 1998). The exact reasons for this are not exactly known, but it may have to do with sexual dimorphism in immune status.

As these animals grow and develop so does their immune system. Evidence exists showing disparities between neonatal and adult immune parameters (Jain, 1993; Knowles et al., 2000) and functions (Zwahlen and Roth, 1990; Lee and Roth, 1992; Kampen et al., 2006). Few studies exist with large sample sizes quantifying immune indices in neonatal and growing cattle. This is especially true for immune parameters of Brahman cattle and other beef breeds, as the majority of the research is carried out in dairy cattle. Since dairy cattle are managed differently than beef cattle their immune cell parameters may vary. Therefore our objective was to examine immune indices in calves subjected to prenatal stress during the neonatal stage and to follow these calves through weaning. This will establish reference data for calf immune indices through the age of weaning.

## Materials and Methods

### *Experimental Design*

Two groups of Brahman Cows (total N= 96) were selected for this study, a prenatally stressed group, (PNS, N= 48), and a control group, (CONT, N= 48) based on their mature temperaments as adult cows. The two groups of cows were matched for

temperament, age and parity. The temperament groupings consisted of calm, intermediate, and temperamental cows. Each cow was classified into one of three temperament groups: 1) calm, 2) intermediate, 3) temperamental (Stahringer et al., 1990). Both groups of cows were kept in a pasture until being transported. The prenatally stressed group experienced transport for 2 h each on d 60, 80, 100, 120, and 140 ± 5 d of gestation. Calves born to the two groups of cows were utilized in this study and are referred to as control (CONT) and prenatally stressed (PNS) calves.

***The First 24 h***

Calves were sampled at least 6 h after ingesting colostrum and within 24 h of birth. Measurements on the calf included quantification of concentrations of serum cortisol along with a sample for a complete blood count (CBC). Calf blood was collected via jugular vein puncture into BD vacutainer tubes containing either EDTA for the CBC samples or no additives for the serum samples.

***14 and 28 days of Age***

At 14 d and 28 d of age, each calf was brought up through the chute for blood sample collection. The calf had its exit velocity (EV) measured and a pen score (PS) assigned. The exit velocity (m/s) is calculated by recording the length of time it takes a calf to exit a working chute and traverse a set distance of 1.83 m (Burrow, 1988; Curley et al., 2008). Measurements of EV were taken with infrared sensors placed at the exit of the working chute (Farmtek Inc., North Wylie, Texas). The pen score is assessed by an unbiased observer based on the calf's behavior and aggressiveness during interactions with an observer and other calves while in a pen (Burdick et al., 2010; Burdick et al.,

2011a; Burdick et al., 2011b). Blood collection on the calf included samples for measurements of serum cortisol concentrations and CBC.

***28 days Prior to Weaning through Weaning***

At 28 d prior to the date of weaning (after referred to as -28 d) and at weaning (after referred to as 0 d), blood samples were collected, EV, PS and temperament scores were recorded. Blood samples were collected through jugular vein puncture to measure serum cortisol concentrations and a CBC.

*Serum Collection*

Blood from the calves was collected and processed to yield serum. The blood was refrigerated overnight at 4 ºC and centrifuged the next morning at 3,000 G for 20-25 min at 6-7˚C. The serum was flash frozen in liquid nitrogen and stored at -80˚C until processing.

*CBC Testing*

Blood samples for CBCs were collected into a 2.5 ml EDTA vacutainer tube via jugular vein puncture within the first 24 h of birth and analyzed using the IDEXX Protocyte Dx Hematology Analyzer (IDEXX Laboratories Inc., Westbrook, ME). The samples were inverted a minimum of 15 times prior to being placed into the machine. The Procyte Dx analyzed the sample and a report per calf sample was recorded. The CBC analysis included cell counts for total white blood cells (WBC), lymphocytes, monocytes, eosinophils, basophils, and neutrophils. CBC analysis of calf blood was again done in the same manner as described on 14 d, 28 d, 28 d prior to weaning (-28 d), at weaning (0 d). All samples were processed within 10 minutes of collection to reduce

clotting, platelet aggregation, and any degradation to the samples. Values for the neutrophil:lymphocyte ratio (NL ratio) were calculated based upon the results obtained from the neutrophil and lymphocyte cell counts. The NL ratio is used as a measurement of stress assessment in animals (Widowski et al., 1989; Davis et al., 2008).

*Cortisol Assay*

Serum concentrations of cortisol were determined from duplicate samples with the use of a single antibody radioimmunoassay (Coat-A-Count Cortisol Kit # TKC05, Siemens Medical Solutions Diagnostics, USA) that utilized antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009; Hulbert et al., 2012). Serum cortisol concentrations were determined by comparison of unknown samples with a standard curve generated from known concentrations of cortisol using Assay Zap software (Biosoft, Cambridge, UK). The minimum detectable cortisol concentrations for this assay were 1.2 ng/mL and the intra- and inter-assay coefficients of variation were 6.4 % and 5.6%, respectively. Data from this assay are presented as concentrations in ng/mL.

*Statistical Analysis*

Data were first analyzed for normalcy using PROC univariate procedures in SAS software (SAS, 2011). The subsequent distributions revealed some data to have non-normal distributions. As such, natural log transformations were applied to the data prior to analysis when needed to correct for non-normalcy. Statistical analysis was done on the transformed data in SAS software (SAS, 2011) using a mixed model with fixed effects of age, sex, and prenatal treatment for the 24 h, 14 d and 28 d analyses. For the

analysis of -28 d and 0 d, the fixed effect of age was replaced with day relative to weaning. A fixed effect of weaning replicate was added along with the calf temperament score as a covariate at -28 d and 0 d. Interactions were included in the model initially, and were subsequently removed if found to be non-significant. The natural log values obtained after analysis were back calculated to yield values with respect to the original data. Data are reported as the least squares means ± standard errors.

**Results**

*24 h Calf CBCs*

At 24 h of age, calf CBC and cortisol analyses were carried out to determine if these were affected by prenatal stress. Calf WBC counts at 24 h tended to be affected by sex, ($P = 0.0668$), with females (15.25 ± 1.05 K/μL) having greater cell counts than males (13.38 ± 1.04 K/μL). The 24 h WBCs were not affected by prenatal stress (PNS) ($P > 0.5$) alone, however there was a tendency (Figure 12, $P = 0.0611$) for a sex by treatment interaction, whereby control (CONT) females had greater WBCs than PNS females. Conversely, the PNS males had greater WBCs than CONT males.

The lymphocytes at 24 h were not affected by sex ($P > 0.9$), as cell counts were 4.3876 ± 0.1956 and 4.3607 ± 0.1762 K/μL for females and males, respectively. No effect of treatment ($P > 0.1$) was observed for the lymphocytes where the cell counts were 4.1887 ± 0.1906 and 4.5596 ± 0.1816 K/μL for the CONT and PNS, respectively. No interactions ($P > 0.5$) affected lymphocyte numbers.

The monocytes, although not affected by prenatal treatment ($P > 0.1$; 0.1884 ± 0.024 and 0.2389 ± 0.023 K/μL, CONT and PNS respectively), tended to be affected by calf sex (Figure 13, $P = 0.0973$), with females having greater monocyte cell counts than the males. No interactions ($P > 0.7$) were shown to affect the monocytes.

Similarly, the neutrophils exhibited a strong tendency for an effect of sex ($P = 0.0506$, Figure 14), with females having the greater cell count compared to males. The 24 h neutrophils also displayed a weak tendency for a sex by treatment interaction ($P = 0.102$), whereby CONT females (12.1013 ± 1.0579 K/μL) had greater cell counts than PNS females (10.4623 ± 0.9022 K/μL). Conversely, the PS males (10.1533 ± 0.9234 K/μL) had numerically higher cell counts than CONT males (8.6964 ± 0.8463 K/μL). There was no effect of treatment on the neutrophils, ($P > 0.9$).

Observations of the eosinophils, yielded no influence of main effects ($P > 0.3$) or interactions ($P > 0.4$) on their cell counts. Eosinophil numbers were 0.0373 ± 0.00688, 0.03873 ± 0.0062, 0.03384 ± 0.0067, 0.0422 ± 0.00639 K/μL, for females, males, CONT and PNS calves, respectively.

The basophils were affected by calf sex ($P < 0.05$, Figure 15) and also displayed a tendency to be affected by prenatal treatment ($P = 0.092$, Figure 16), with the females and the PNS calves having greater cell counts compared to the males and the CONT calves, respectively. No interactions were observed for the basophils, ($P > 0.2$).

Additionally, the neutrophil:lymphocyte ratio (NL ratio) was not affected by sex ($P > 0.1$), although females (2.44 ± 1.088 K/μL) had numerically greater NL ratios than males (2.06 ± 1.079 K/μL). No effect of prenatal treatment ($P > 0.4$) was observed for

NL ratios (2.33 ± 1.086 and 2.158 ± 1.082 K/µL for CONT and PNS calves, respectively). No interactions were observed ($P > 0.2$) affecting the NL ratio.

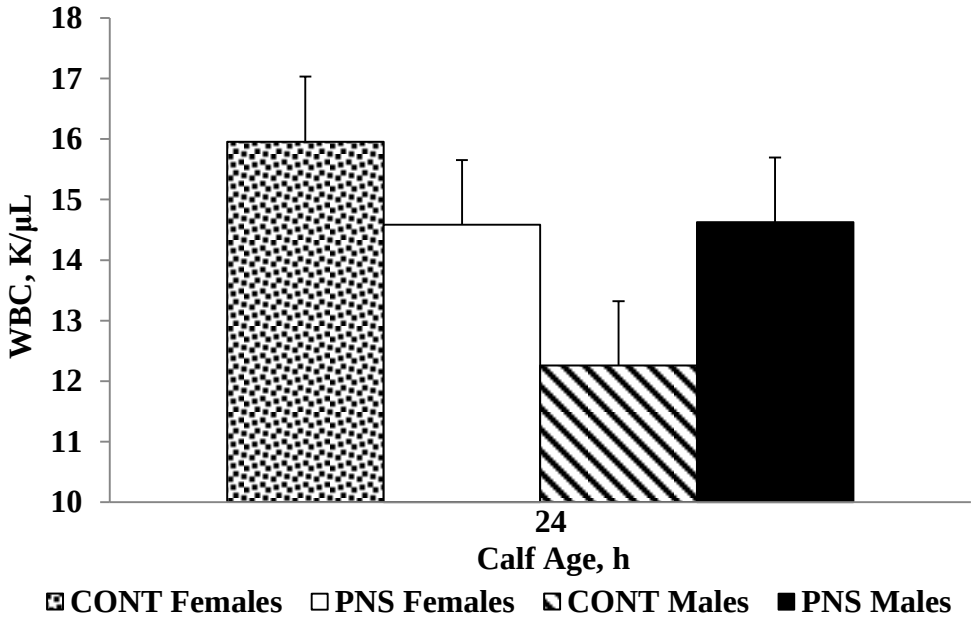


**Figure 12.** Calf white blood cell counts for the sex by treatment interaction at 24 h. The control (CONT) females had the numerically greatest cell counts, ($P = 0.0611$). All the females had numerically greater cell counts than either group of the males ($P = 0.0668$). However, the prenatally stressed (PNS) males had greater cell counts than the CONT males.


**Figure 13.** Calf monocyte cell counts at 24 h. The females had numerically greater cell counts than the males, ($P$ = 0.0973).

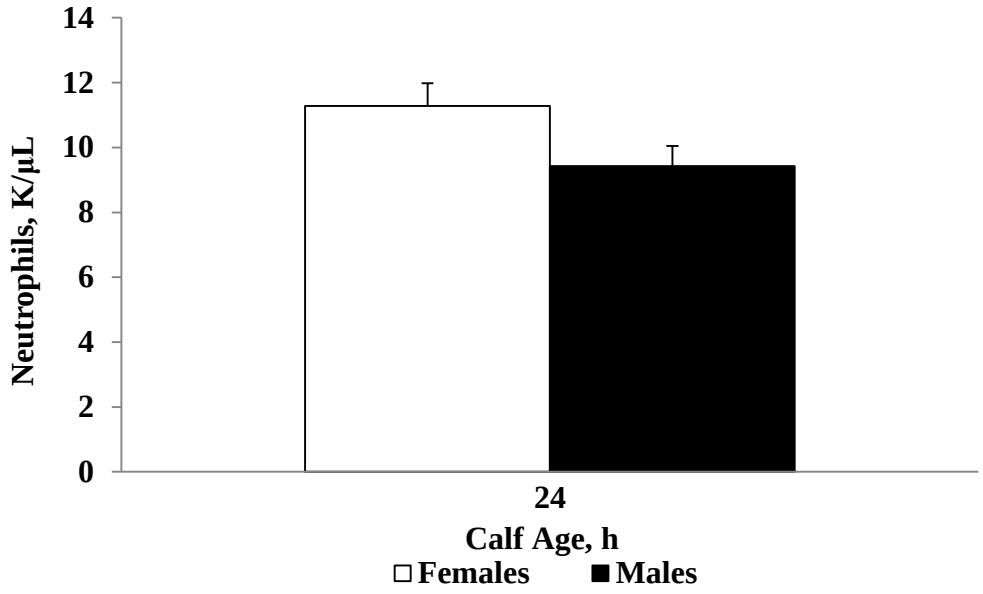


**Figure 14.** Calf neutrophil cell counts at 24 h. The females had numerically greater cell counts than the males, ($P = 0.0506$).


**Figure 15.** Calf basophil cell counts by sex at 24 h. The females had greater cell count than the males, ($P$ = 0.0454).

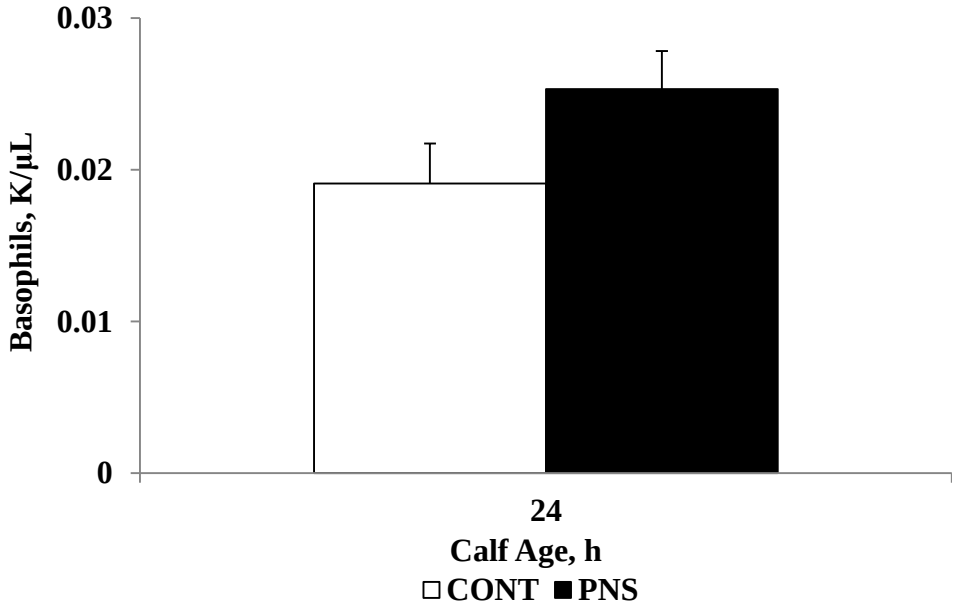


**Figure 16.** Calf basophil cell counts by prenatal treatment at 24 h. The prenatally stressed (PNS) calves had tendencies for greater cell counts than control (CONT) calves, ($P = 0.092$).

*24 h Calf Cortisol*

Cortisol concentrations at 24 h were affected by prenatal treatment (Figure 17, $P < 0.03$), with the PNS calves having 10 ng/mL greater cortisol concentrations than the CONT calves. Cortisol was not affected by sex ($P > 0.9$) as concentrations were 59.69 ± 3.25 and 59.92 ± 2.92 ng/mL for females and males, respectively. No interactions ($P > 0.1$) affecting cortisol concentrations were observed.

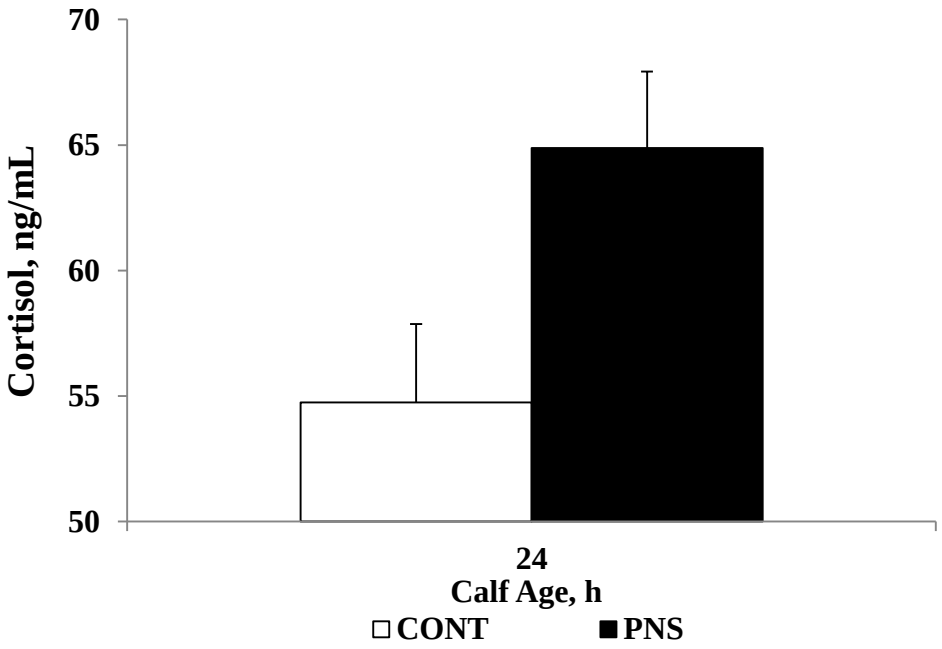


**Figure 17.** Calf cortisol concentrations vary by prenatal treatment at 24 h. The prenatally stressed (PNS) calves had greater cortisol concentrations than control (CONT) calves, ($P < 0.03$).

*14 d and 28 d Calf CBCs*

The calves' CBCs and cortisol concentrations were examined at 14 d and 28 d of age. The WBCs were not affected by sex ($P > 0.4$; 13.96 ± 1.037 and 13.43 ± 1.033 K/μL for females and males, respectively) or by treatment ($P > 0.4$), as CONT had 13.94 ± 1.035 K/μL, while PNS had 13.45 ± 1.036 K/μL. However, there was an effect of age ($P = 0.0018$, Figure 18) and a treatment by age interaction ($P = 0.0482$, Figure 19) observed for the WBCs. The WBCs counts increased from 14 d to 28 d after birth. Concerning the age by treatment interaction, differences in WBC were seen at 28 d, where CONT calves had greater WBC numbers than PNS calves.

The lymphocytes were only affected by age ($P = 0.0001$, Figure 20), with greater cell counts seen at 28 d than at 14 d. No effects of sex ($P > 0.2$) or treatment ($P > 0.8$) were observed as females had cell counts of 6.9809 ± 0.2972 K/μL while male counts were 7.4251 ± 0.2648 K/μL. The CONT calves had cell counts of 7.163 ± 0.281 K/μL, and the PNS calves' lymphocyte numbers were 7.2428 ± 0.2819 K/μL. There were no interactions ($P > 0.3$) affecting the lymphocytes at 14 and 28 d.

Examination of the monocytes showed them to only be affected by age ($P < 0.0001$), whereby the greatest cell counts occurred at 28 d compared to 14 d, (Figure 21). Both males and females had similar monocyte cell counts, (1.17 ± 0.032 and 1.11 ± 0.028 K/μL for females and males, respectively), as sex was not significant ($P > 0.4$). Prenatal treatment ($P > 0.9$) was not a factor affecting monocyte counts, as both CONT and PNS had cell counts of 1.14 ± 0.03 K/μL. No interactions were shown to affect monocyte numbers ($P > 0.9$).

As for the neutrophils, they were not affected by prenatal treatment ($P > 0.3$) or age ($P > 0.8$) alone. Instead, a treatment by age interaction ($P = 0.0278$) was observed for the neutrophils (Figure 22), with all calves having similar neutrophil counts at 14 d, but at 28 d, CONT calves had greater neutrophil counts than the PNS calves. Additionally, there was a tendency for sex ($P = 0.0908$) to affect neutrophil cell counts (Figure 23), with females having numerically greater cell counts than males.

No factors or interactions significantly affected eosinophil counts, ($P > 0.2$). Eosinophil cell counts were 0.048 ± 0.006 and 0.056 ± 0.006 K/μL at 14 and 28 d, respectively. Similarly, the CONT eosinophils were 0.049 ± 0.007 K/μL and the PNS eosinophils were 0.056 ± 0.007 K/μL. The female and male eosinophil numbers were also similar with 0.057 ± 0.007 and 0.047 ± 0.006 K/μL for females and males, respectively.

For the basophils, a strong tendency for sex ($P = 0.0579$) to affect the cell counts was found (Figure 24), where females had numerically greater basophil cell counts compared to males. No other main effects of age ($P > 0.9$; 0.0145 ± 0.0015 K/μL for 14 d and 0.0147 ± 0.0015 K/μL for 28 d) or treatment ($P > 0.4$; 0.0154 ± 0.002 K/μL for CONT and 0.0138 ± 0.002 K/μL for PNS) or interactions ($P > 0.6$) were seen for the basophil cell counts. Interesting to note, cattle are known to have extremely low basophil numbers, so results of 0 K/μL may be obtained when using a hematological analyzer or manual method of counting (Jones and Allison, 2007).

The NL ratio was affected by sex ($P = 0.0347$), with females having greater NL ratios than males (Figure 25). No effects of treatment ($P > 0.3$), age ($P > 0.1$), or

interactions ($P > 0.1$) were observed to affect the NL ratio. Despite the lack of significance, CONT calves (0.82 ± 0.03 K/µL) had a numerically greater NL ratio than the PNS (0.75 ± 0.03 K/µL) calves. Also the NL ratio was greater at 14 d (0.81 ± 0.02 K/µL) than at 28 d (0.76 ± 0.02 K/µL).

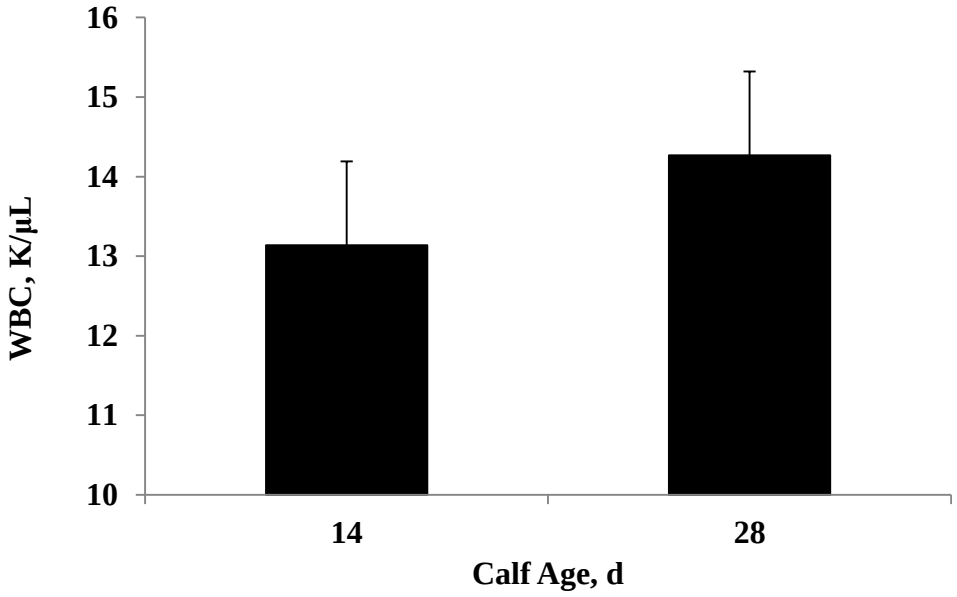


**Figure 18.** Calf white blood cell counts at 14 d and 28 d of age. The cell counts increased significantly from 14 d to 28 d, ($P = 0.0018$).

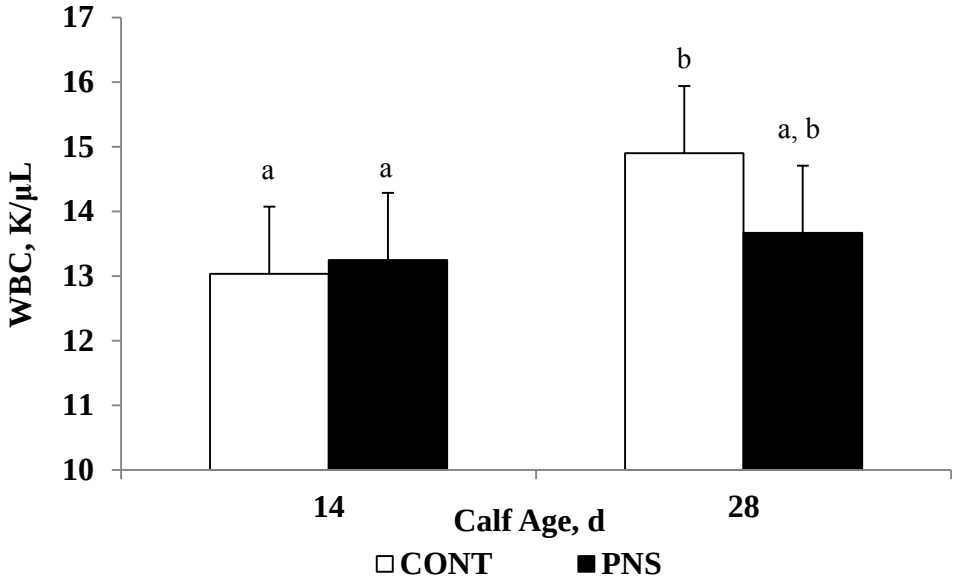


**Figure 19.** Calf white blood cell counts by prenatal treatment and calf age. The WBCs at 14 d were similar for both control (CONT) and prenatally stressed (PNS) calves. However at 28 d, the CONT, had significantly greater cell counts, ($P = 0.0482$) compared to PNS. Bars separated by different letters had means that differed significantly, ($P < 0.05$).

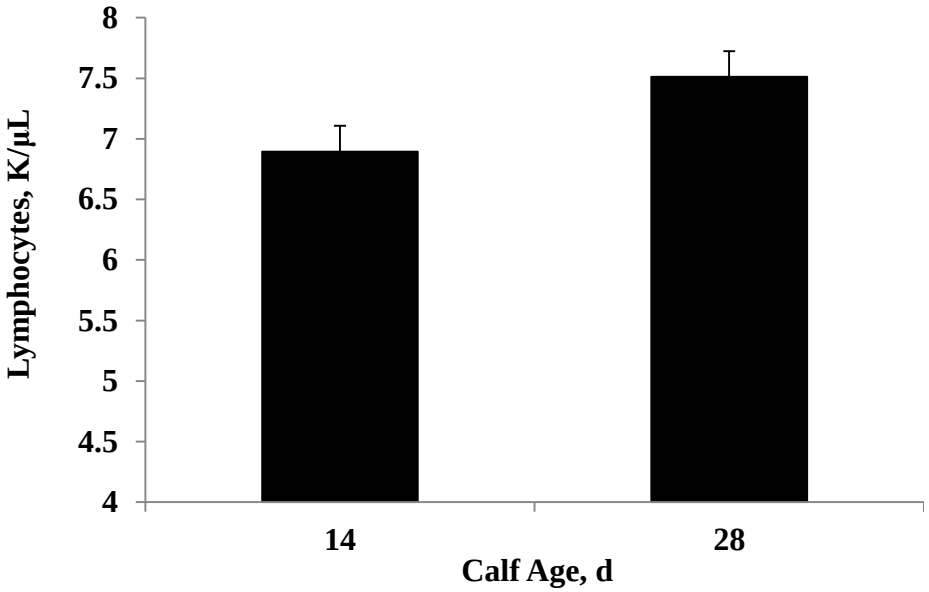


**Figure 20.** Calf lymphocyte cell counts at 14 d and 28 d. The lymphocytes in all calves increased from 14 to 28 d, ($P < 0.0001$).

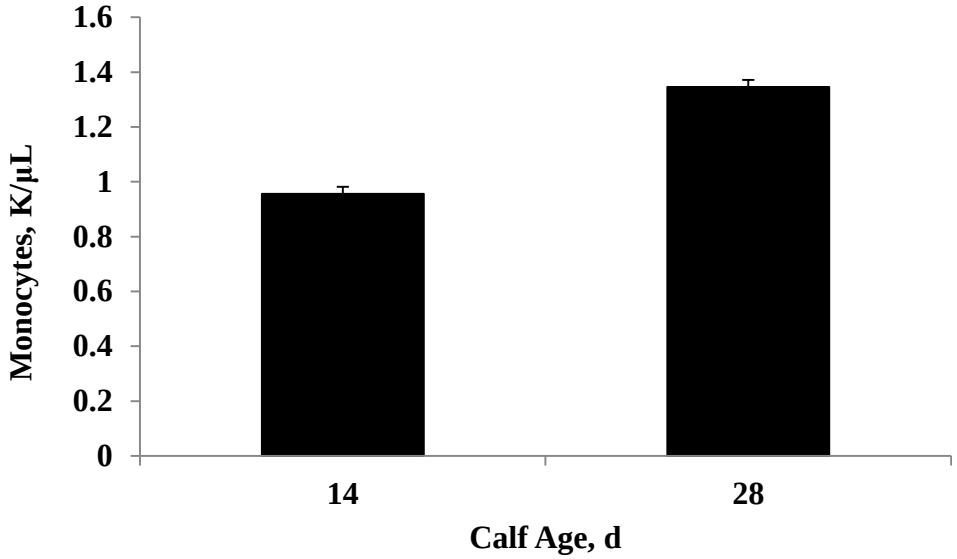


**Figure 21.** Calf monocyte cell counts at 14 d and 28 d. The monocyte numbers increased from 14 to 28 d, ($P < 0.0001$).

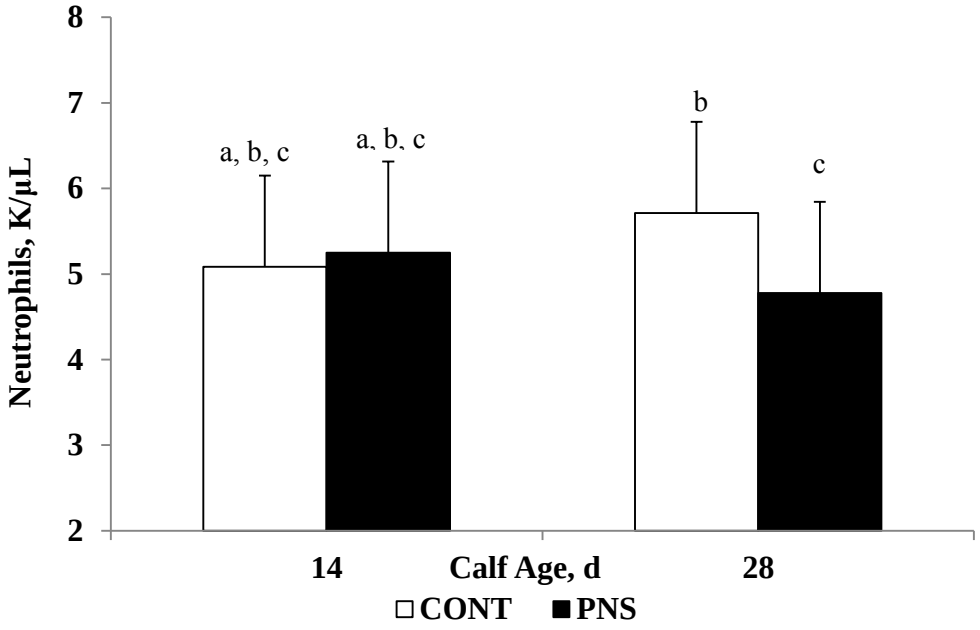


**Figure 22.** Calf neutrophil cell counts by age and prenatal treatment at 14 d and 28 d. The calf age alone did not affect neutrophil cell counts, ($P > 0.05$). However at 14 d, the control (CONT) and the prenatally stressed (PNS) calves had similar cell counts, but at 28 d, the CONT had greater cell counts, ($P = 0.0278$) than PNS calves. Bars separated by different letters have significantly different means, ($P < 0.05$).


**Figure 23.** Calf neutrophil cell counts by sex pooled over 14 d and 28 d. The females had numerically greater cell counts than the males, ($P$ = 0.0908).

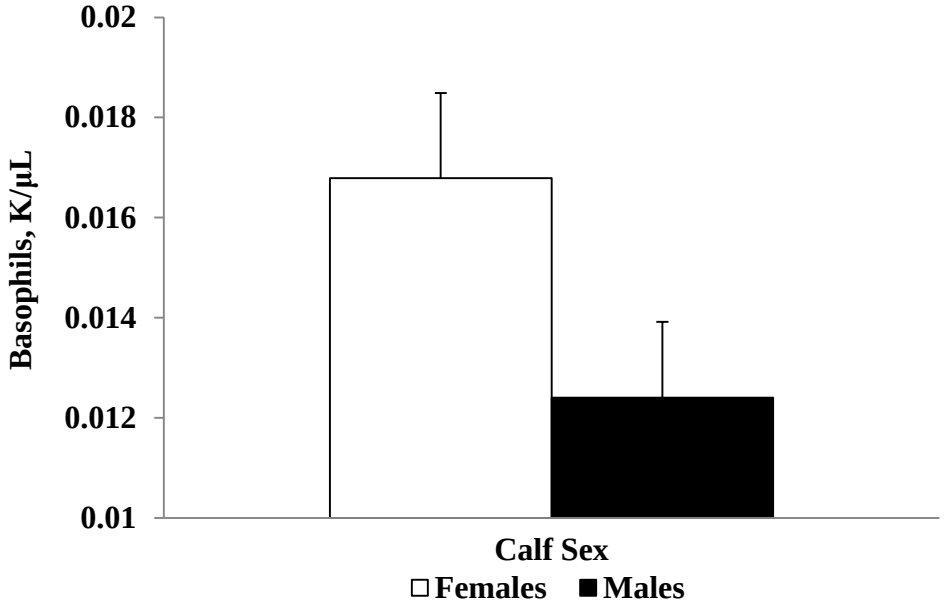


**Figure 24.** Calf basophil cell counts by sex pooled over 14 d and 28 d. The females had numerically greater cell counts than the males, ($P = 0.0579$).

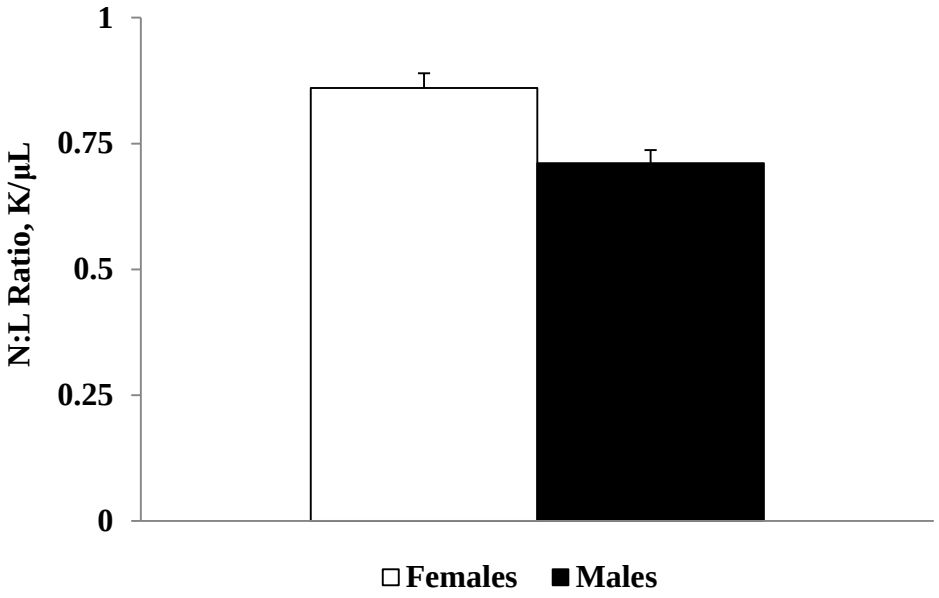


**Figure 25.** Calf neutrophil:lymphocyte ratios (NL ratio) by sex pooled over 14 d and 28 d. The females had greater NL ratios ($P < 0.04$) than the males.

*14 d and 28 d Calf Cortisol*

The cortisol concentrations at 14 d and 28 d were affected by sex ($P = 0.0102$), with females having greater cortisol concentrations than males (Figure 26). No effects were found for age ($P > 0.3$), as concentrations were 12.76 ± 1.06 ng/mL at 14 d and 12.06 ± 1.06 ng/ mL at 28 d. There was no effect of prenatal treatment ($P > 0.6$) as cortisol concentrations were 12.12 ± 1.07 ng/mL for CONT and 12.70 ± 1.07 ng/mL for PNS calves. No interactions ($P > 0.1$) affected cortisol concentrations.

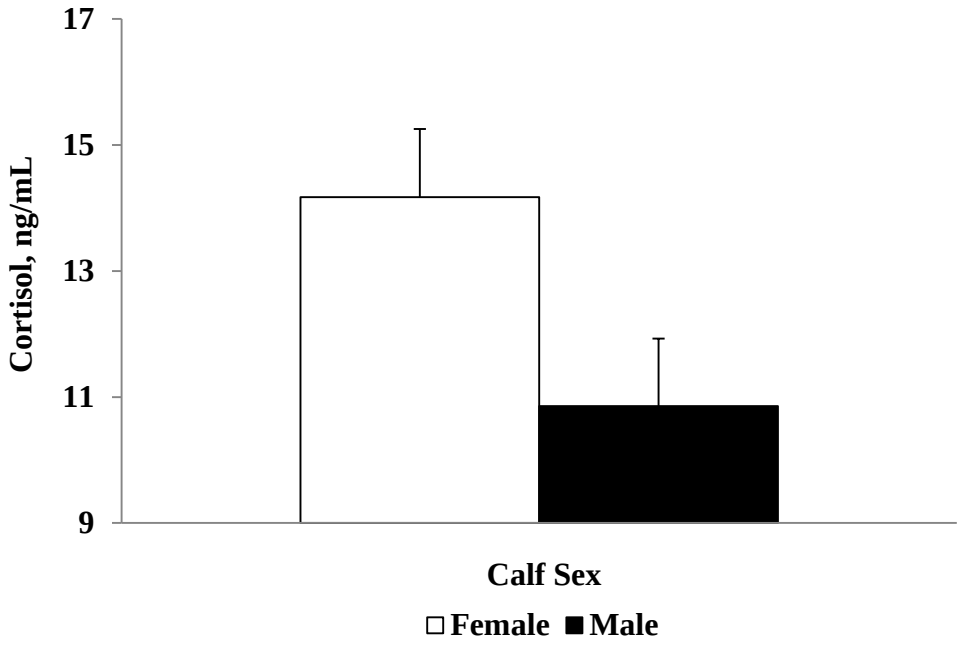


**Figure 26.** Calf cortisol concentrations by sex pooled across 14 d and 28 d. The females had greater ($P < 0.02$) cortisol concentrations than the males.

*-28 and 0 d Relative to Weaning Calf CBCs*

At -28 d and 0 d relative to weaning, analyses of calves' CBC and cortisol concentrations were carried out. The weaning temperament was included in these analyses as a covariate and the d relative to weaning was substituted for calf age as a fixed effect. The WBCs were affected by d relative to weaning ($P < 0.0001$), with cell counts being greater at -28 d than at weaning (Figure 27). There were no effects of treatment ($P > 0.4$) as CONT cell counts were 14.10 ± 0.51 K/μL, while PNS cell counts were 13.52 ± 0.50 K/μL. Nor was there an effect of sex ($P > 0.5$), with female cell counts being 13.61 ± 0.53 K/μL and 14.01 ± 0.49 K/μL for males. Temperament did not ($P > 0.2$) account for much of the variability in the WBCs.

The lymphocytes were affected by d relative to weaning ($P < 0.0001$), with greater cell counts being observed at -28 d (Figure 28) than at 0 d. There was no effect of treatment ($P > 0.5$, CONT numbers were 8.29 ± 1.04 K/μL and PNS numbers were 8.03 ± 1.04 K/μL), nor did temperament ($P > 0.1$) account for significant variability of the lymphocyte cell counts. However, there was a weak tendency for sex ($P = 0.1052$) to affect the lymphocyte counts, where female cell counts were 7.75 ± 1.05 K/μL and males were 8.58 ± 1.04 K/μL.

Data for the monocytes revealed both d relative to weaning ($P = 0.0034$; Figure 29) and sex ($P = 0.0312$; Figure 30) to affect the cell counts, with greater cell counts occurring at 0 d compared to -28 d, and in females compared to males. There was no effect of treatment ($P > 0.7$), as CONT had monocyte counts of 1.22 ± 0.033 K/μL and

PNS had counts of 1.26 ± 0.32 K/μL. No interactions ($P > 0.5$) affected monocyte cell counts.

The neutrophils were only affected by d relative to weaning ($P = 0.0005$), with the greater cell counts observed on -28 d (Figure 31) than at 0 d. No effects of treatment ($P > 0.6$) were observed, although the CONT (3.8 ± 0.17 K/μL) had numerically greater neutrophil counts than PNS (3.5 ± 0.17 K/μL). Sex ($P > 0.2$) was not a factor affecting the neutrophils, as both female (3.7 ± 0.18 K/μL) and male (3.6 ± 0.16 K/μL) cell counts were similar.

Although the eosinophils were not affected by d relative to weaning ($P > 0.1$), the numerically greater cell counts were observed at 0 d (0.321 ± 0.03 K/μL) compared to – 28 d (0.27 ± 0.3 K/μL). Similar findings are shown for sex ($P > 0.4$) and prenatal treatment ($P > 0.3$) where almost the same eosinophil counts of 0.31 ± 0.04 K/μL for females, 0.27 ± 0.04 K/μL for males, 0.27 ± 0.04 K/μL for CONT, and 0.32 ± 0.04 K/μL for PNS were recorded. No interactions affected eosinophil counts ($P > 0.1$).

Similarly, the basophils were not affected by the d relative to weaning ($P > 0.2$; 0.009 ± 0.002 K/μL at -28 d and 0.006 ± 0.002 K/μL at 0 d), sex ($P > 0.6$; 0.008 ± 0.002 K/μL for females and 0.007 ± 0.002 K/μL for males), or prenatal treatment ($P > 0.5$; CONT had 0.009 ± 0.002 K/μL, and PNS had 0.006 ± 0.002 K/μL). Nor interactions were detected ($P > 0.2$) for the basophils.

Examination of the NL ratio revealed no effect of prenatal treatment ($P > 0.6$), as the CONT ratio was 0.47 ± 0.02 K/μL and the PNS ratio was 0.45 ± 0.02 K/μL. However, the NL ratio was affected by sex ($P = 0.0459$; Figure 32), whereby the females

had greater NL ratios than males. There was a tendency for the weaning replicate ($P$ = 0.0893) to affect the NL ratio, with replicate 1 (0.49 ± 0.02 K/µL) having greater NL ratios than replicate 2 (0.43 ± 0.02 K/µL). The d relative to weaning ($P > 0.6$) alone did not affect the NL ratio, although an interaction for replicate by d relative to weaning ($P$ = 0.003) was observed, where both replicates had similar values on -28 d, but on 0 d, replicate 1 had greater ratios than replicate 2 (Figure 33). Additionally, temperament ($P$ = 0.0605) tended to account for some of the variability in the NL ratio.

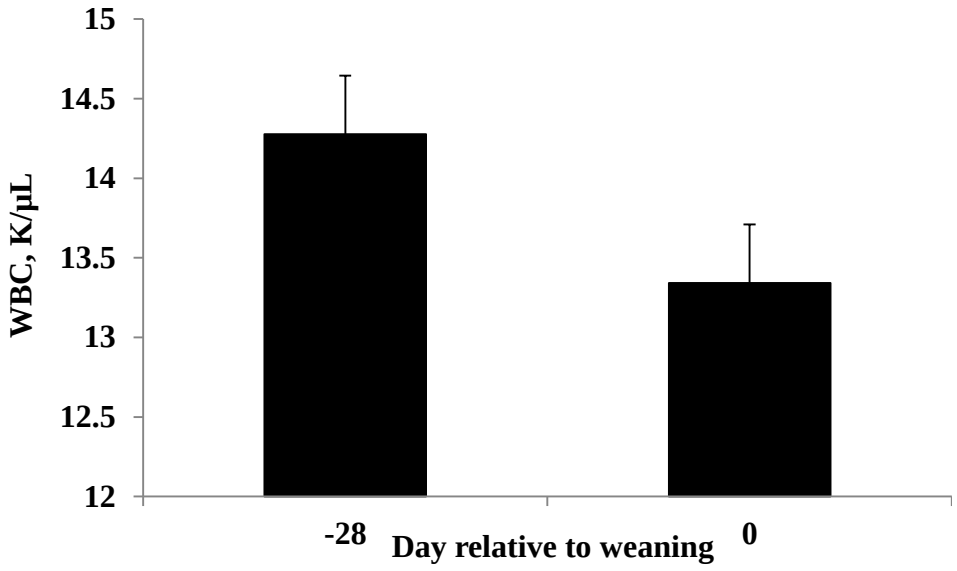


**Figure 27.** Calf white blood cell (WBC) counts at -28 d and 0 d. The WBCs decreased from -28 d to 0 d, ($P < 0.0001$).

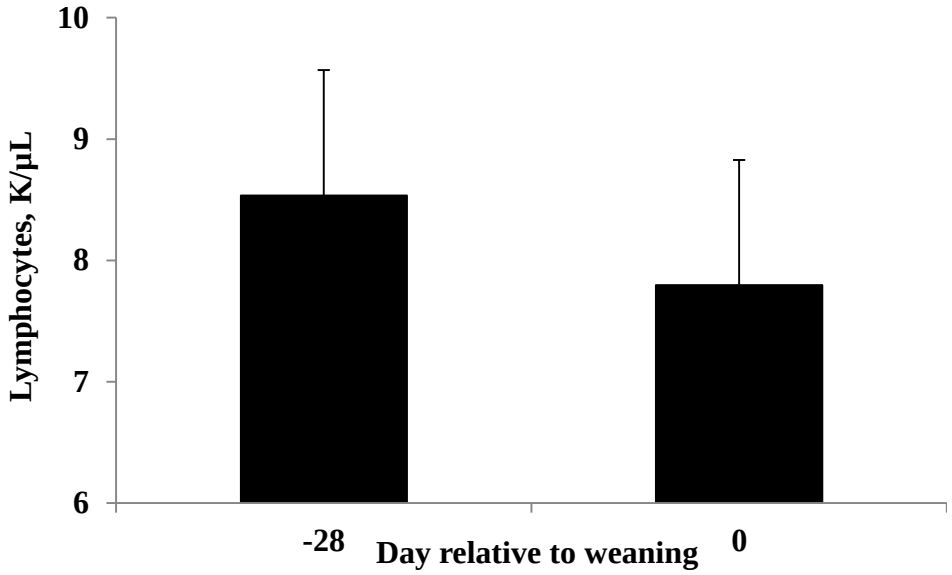


**Figure 28.** Calf lymphocyte cell counts at -28 d and 0 d. The cell counts were greater at - 28 d than they were at 0 d, ($P < 0.0001$).

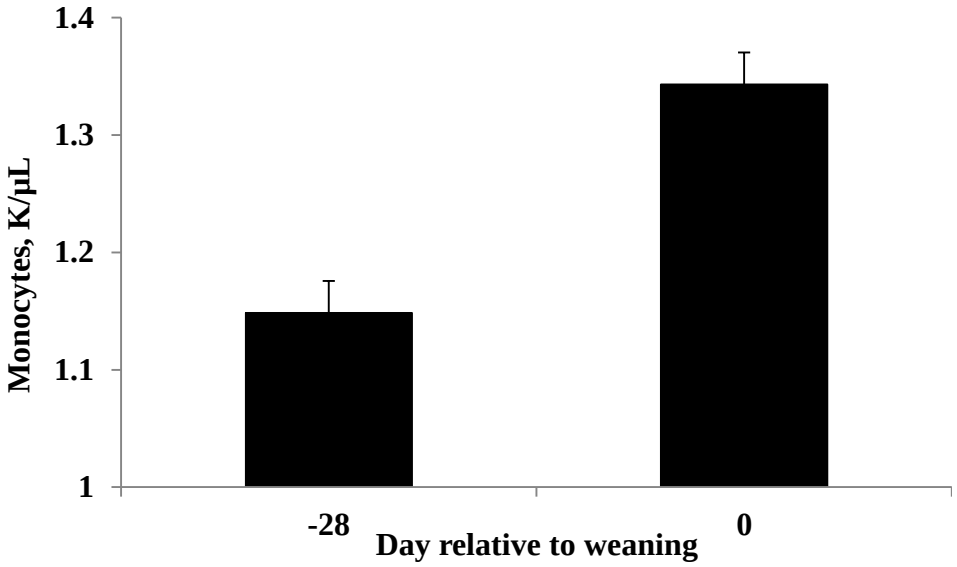


**Figure 29.** Calf monocyte cell counts at -28 d and 0 d. The cell counts increased from -28 to 0 d, ($P < 0.01$).

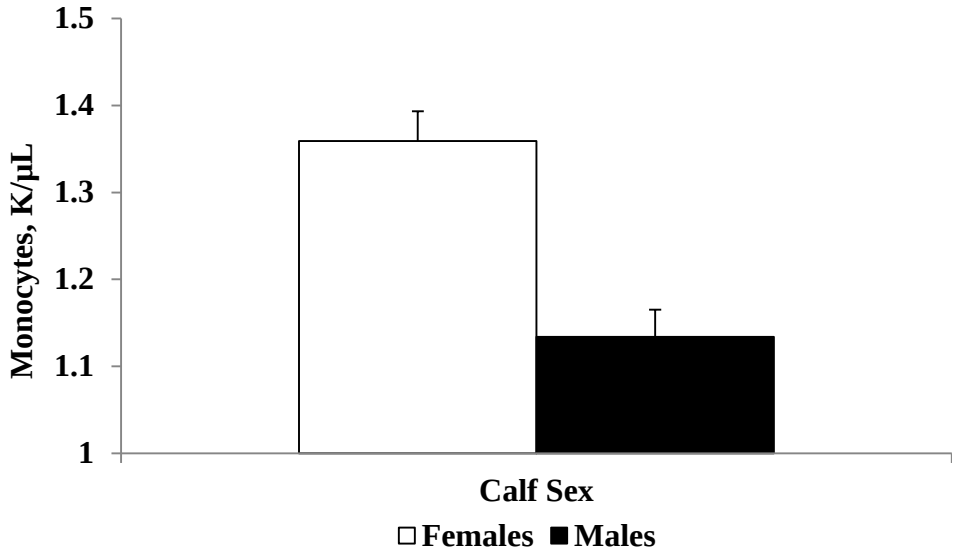


**Figure 30.** Calf monocyte cell counts by sex pooled across -28 d and 0 d. The females had greater monocyte counts than the males ($P < 0.04$).

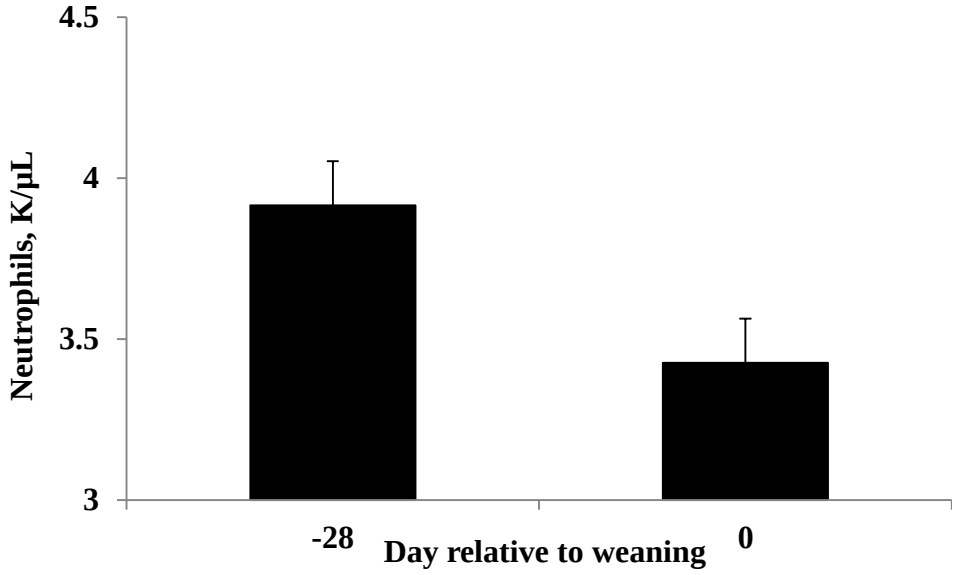


**Figure 31.** Calf neutrophil cell counts at -28 d and 0 d. The neutrophil counts decreased from -28 d to 0 d ($P < 0.001$).

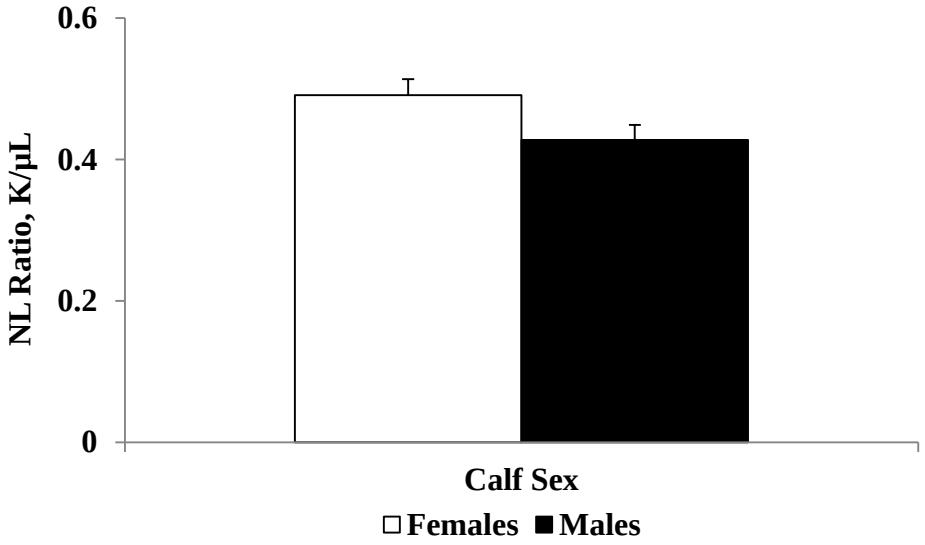


**Figure 32.** Calf neutrophil:lymphocyte (NL) ratio by sex pooled over -28 d and 0 d. The females had a greater NL ratio than males ($P < 0.05$).

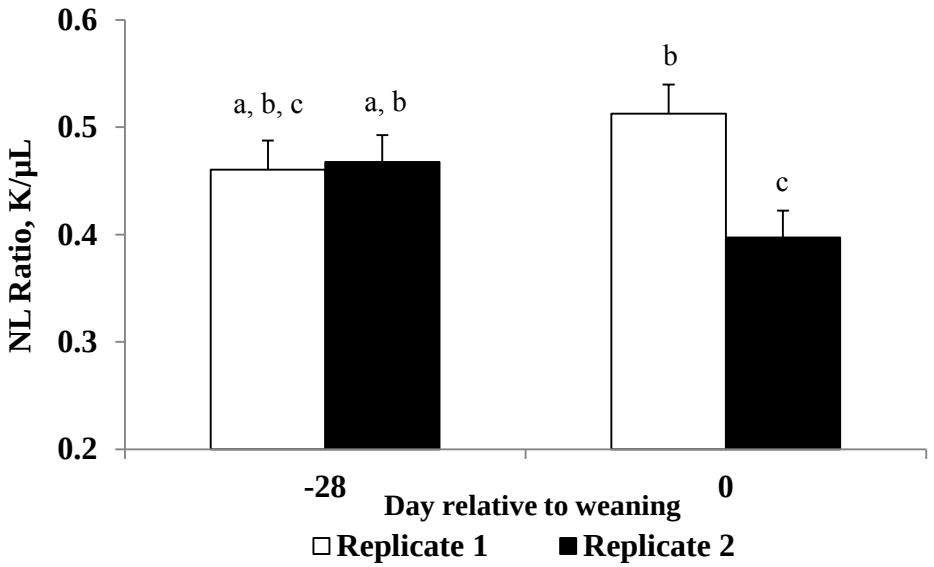


**Figure 33.** Calf neutrophil:lymphocyte (NL) ratio by replicate and day relative to weaning. At -28 d, both replicates had similar NL ratios, while at 0 d, replicate 1 had a greater NL ratio than replicate 2 ($P < 0.005$). Bars separated by different letters had means that significantly differed, ($P < 0.05$).

*-28 d and 0 d Relative to Weaning Calf Cortisol*

At -28 d, cortisol concentrations were less than at day 0 d (Figure 34), evidenced by the tendency for d relative to weaning ($P = 0.0789$) to affect cortisol concentrations. Sex of the calf ($P = 0.0042$) was highly influential on cortisol concentrations, as females had greater cortisol concentrations than males (Figure 35). Additionally, calf temperament accounted for a large amount of the variability in cortisol concentrations ($P < 0.0001$). There was no effect of prenatal treatment ($P > 0.5$), as CONT concentrations were 11.74 ± 1.06 ng/mL and PNS concentrations were 12.30 ± 1.06 ng/mL. No interactions ($P > 0.4$) were observed to affect cortisol concentrations.

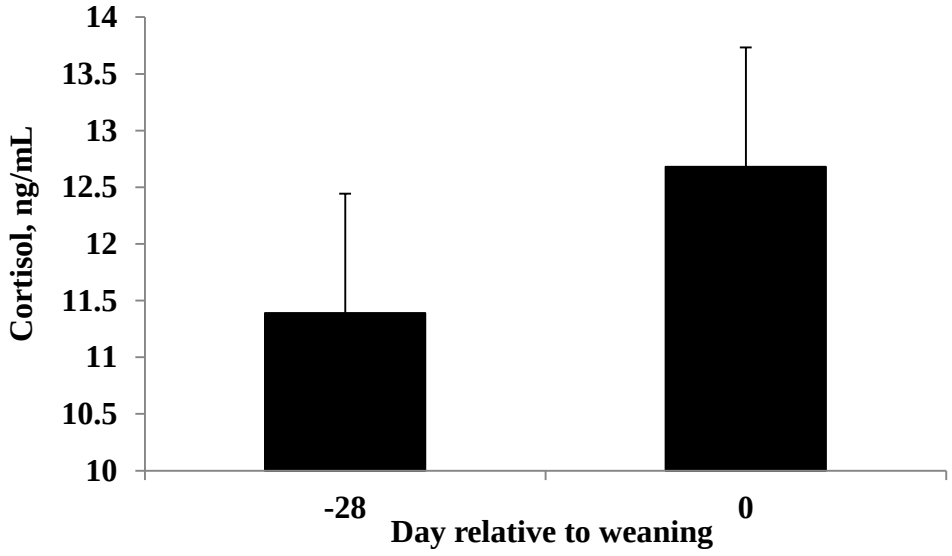


**Figure 34.** Calf cortisol concentrations at -28 d and 0 d. The calf cortisol concentrations were less at -28 d and numerically increased at 0 d ($P = 0.0789$).

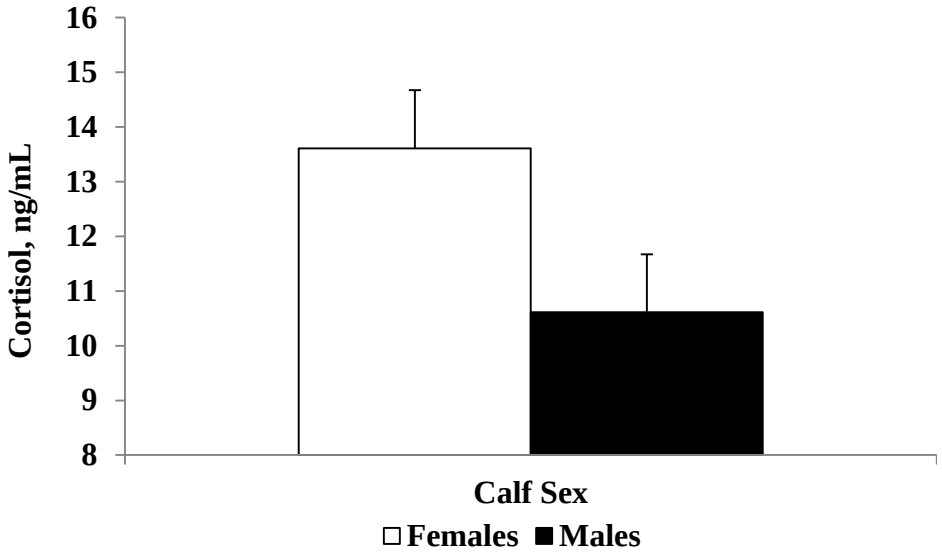


**Figure 35.** Calf cortisol concentrations by sex pooled across -28 d and 0 d. The females had greater cortisol concentrations than the males ($P < 0.005$).

**Discussion**

The effects of prenatal stress were investigated on both immune indices and cortisol concentrations from birth through the age of weaning. At birth, variation in immune indices is the result of sexual dimorphism between males and females, with females having numerically greater neutrophil and monocyte cell counts than males. This can help explain why more neonatal deaths are attributed to males.

These differences in immune indices are mirrored in recent work showing female Brahman heifers to elicit greater cytokine responses to a CRH challenge compared to bulls (Hulbert et al., 2012). Hematological analysis in adult and kid West African Dwarf goats demonstrated females to have greater neutrophil cell counts and percentages than males (Opara et al., 2010). Our observations of sexual dimorphism in immune indices are contrary to prior reports by Mohri et al, (2007), Tennant et al. (1974), and Adams et al. (1992), who did not see differences between male and female dairy and beef calves. The larger sample size in our study may have allowed us to demonstrate these trends and significant differences. Research in several breeds of pigs reported a lack of sexual dimorphism in immune cell variables (Sutherland et al., 2005).

Additionally, we observed tendencies for interactions between sex and prenatal treatment for the neutrophils and the basophils, both of which are essential cells in innate immune defense. Interestingly, prenatal stress increased male immune indices, while decreasing female indices. This suggests within 24 h of birth, prenatal stress may enhance male immune cell counts, while simultaneously hindering immune cell counts

in females. Furthermore, it demonstrates that effects of prenatal stress may be mediated by sex in cattle.

Immune indices are affected by breed, calf age, gender, and health status of the animal. When comparing means for immune cell counts with those of known reference ranges from Jain (1993), our 24 h, 14 d and 28 d values fell into their reference ranges, with the exception of the total WBCs, neutrophils, and NL ratio, all of which were greater than their reference ranges at each age. This can be attributed to the greater neutrophil cell counts that we observed, as they would increase both the total WBCs and the NL ratio. All of the immune indices measured, with the exceptions of eosinophil and basophils were altered by calf age. Age related changes are not surprising as the immune system of the calves is continuing to develop as they grow and mature. All of our total WBCs fell within the age related reference ranges given by Jezek et al, (2011). Observations of 0 K/μL for both basophils and eosinophils were reported in other studies (Eberhart and Patt, 1971; Tennant et al., 1974; Knowles et al., 2000), and are values included in reference ranges for these immune cells (Jain, 1993).

Our WBC data agrees with previous findings in Holstein (15.33 ± 5.81 K/μL) and beef calves (13.99 ± 5.73 K/μL) at birth and Holstein calves at 15 d (12.09 ± 2.94) (Adams et al., 1992; Mao et al., 1994). A study examining Norwegian Red calves for the first 6 months of life observed lower total WBCs in the range of 9.4-12.0 K/mL, which agreed with adult Norwegian Reds' reference ranges (Brun-Hansen et al., 2006). Their study utilized dairy calves and housed them indoors. The difference in housing is

likely the reason for disparity in total WBCs and other differential cell counts from our study as their calves and adult cows probably encountered less pathogens indoors.

The NL ratio was greatest at birth (2.06 K/μL for males, and 2.44 K/μL for females) and continuously decreased with age, where it stabilized from -28 to 0 d relative to weaning, (0.49 K/μL and 0.43 K/μL for females and males, respectively). These are similar to prior observations where a NL ratio of 2.8 was found at 1 d of age in calves (Tennant et al., 1974). Cattle unlike humans and dogs, have more lymphocytes than neutrophils (Moreira da Silva et al., 1994). Contrarily, at birth this is reversed with neutrophil cell counts being greater, as observed in our study and several others (Adams et al., 1992; Menge et al., 1998; Knowles et al., 2000; Brun-Hansen et al., 2006). These values normalize within the first 2-3 weeks, and can likely be attributed to high cortisol concentrations at birth.

At birth cortisol concentrations were high as expected and decreased by 14 d where they remained similar through the age of weaning. Our findings for CONT calves at birth are in agreement with previously reported values (Eberhart and Patt, 1971; Cabello, 1980). However, the cortisol concentrations we observed at 14 d (12.8 ± 1.06 ng/mL) and 28 d (12.06 ± 1.06 ng/mL), were greater than the 4.9 ± 1.7 ng/mL and 7.5 ± 2.8 ng/mL that Cabello (1980), reported on 15 and 20 d of age, respectively.

Interestingly, prenatal stress significantly increased calf cortisol concentrations by 10 ng/mL at birth. Similarly, prenatal transportation stress in goats tended to yield increases in kid cortisol concentrations at birth (Duvaux-Ponter et al., 2003) and lambs at 25 d of age (Roussel et al., 2004). These results are in contrast to Lay et al.'s previous

reports that prenatal transportation stress did not result in differences in calf cortisol concentrations taken at the time of birth (1997a). Despite the fact that calves experienced the same prenatal stressors in these studies, the small sample size and the fact that calves were delivered by cesarean section in the previous study may account for the lack of difference in cortisol concentrations.

Based on the data at each sampling age from 14 d through weaning, it is evident that sexual dimorphism in cortisol concentrations exists, whereby females exhibit greater cortisol concentrations than males. These findings confirm previous work demonstrating sexual dimorphism in cattle (Henricks et al., 1984; Hulbert et al., 2012), goat (Duvaux-Ponter et al., 2003) cortisol and rat corticosterone (Stefanski and Grüner, 2006) responses. The differences in cortisol/corticosterone responses between males and females are believed to be due to the concentrations of sex hormones, testosterone and estrogen (Brown et al., 2008). Additionally, prenatal stress elicits alterations in calf serum cortisol concentrations, with PNS calves displaying greater amounts of cortisol.

Previous work has demonstrated variations in cortisol concentrations to persist as prenatally stressed (Lay et al., 1997b) calves and rats (Barbazanges et al., 1996) exhibited prolonged cortisol secretion. Our data reveal calf immune parameters vary by age and gender, suggesting future reference ranges for immune indices to include ranges for males and females. Investigations into the concentrations of immunoglobulins and cortisol in colostrum and milk from these cows may help to elucidate some of the reasons behind the differences we have seen in our data. Additional research should consider if the differences in immune indices seen in the prenatally stressed calves

continue as the calves grow, if they change, or if they also are indicative of differences in immune functions. Specifically, investigations into cytokine production and response along with quantifications of gene expression of immune cell receptors may help further elucidate the reasons behind these differences. Moreover, the way in which these prenatally stressed animals respond to future stressors and immune challenges may also help shape future management practices and decisions.

Few studies have examined transgenerational effects of prenatal stress in livestock species. Transgenerational effects of prenatal stress and maternal nutrition have been reported for laboratory animals (Bertram et al., 2008; Schöpper et al., 2012). It would be beneficial to investigate immune indices and function in a future generation of these prenatally stressed calves to see if these changes span generations, as such knowledge could create an even greater impact on neonatal health.

# CHAPTER IV

# INFLUENCE OF PRENATAL STRESS ON NEUTROPHIL FUNCTION AT WEANING

## Introduction

Although a natural part of an animal's life, weaning is a stressful event for juvenile animals, as it involves severing the maternal bond. Evidence in support of the stressfulness of weaning is seen both behaviorally and physiologically (Malinowski et al., 1990; Pollard et al., 1993; Price et al., 2003; Turner et al., 2003; Kojima et al., 2008). Observations of calves abruptly separated from their dams at weaning have shown increased vocalizations, time spent walking and lying down, along with decreased time eating (Price et al., 2003). The greatest differences in these behaviors compared to control calves who remained with their mothers was observed during the first 3 d after weaning, with the largest disparities occurring at 2 d post weaning (Price et al., 2003). Physiological factors demonstrated to be altered by the stress of weaning include increased cortisol (Malinowski et al., 1990; Kojima et al., 2008) and epinephrine responses, reductions in body weights/growth (Pollard et al., 1993) and decreases in ADG (Turner et al., 2003). Additionally, after weaning calves have been known to be more susceptible to respiratory diseases such are BRD along with secondary diseases/infections (Duff and Galyean, 2007; Hodgson et al., 2012).

Stress is known to affect immune function in opposing directions, depending upon the stressor applied, its length of duration, and the individual's ability to respond to the stressor (Dhabhar, 2009). Previous research on weaning stress has yielded interesting results on immune function. Following weaning, lymphocyte counts decreased and coincided with increases in circulating neutrophils leading to a transient neutrophillia and down-regulation of L-selectin in blood neutrophils (Lynch et al., 2010). In several studies where the calves' neutrophil:lymphocyte ratio was calculated, it was consistently seen to be increased, likely due to the increase in neutrophil counts (Hickey et al., 2003; Kim et al., 2011; O'Loughlin et al., 2011).

Neutrophils are known to represent one of the first lines of defense in an animal's body and are responsible for the production of reactive oxidation species (ROS), which are highly toxic to bacteria. With their production of ROS, neutrophils have the potential to cause damage to the animal's own tissues if anti-inflammatory signals are not initiated.

Understanding how stressful events such as weaning impact production animals may help to improve management techniques and overall animal welfare. After being weaned, animals may be vaccinated, commingled, and subjected to transportation (Stanger et al., 2005) and a novel environment upon sale, all of which are known stressors that may further alter immune functions. If animals have a compromised immune system during any of these additional stressors, it may prove detrimental to animal health and ultimately cut into a producer's profits. Conversely, if their immune

system is heightened from acute stress, an informed determination of whether or not to couple any of these procedures can be made.

The aim of this study was to investigate how neutrophil oxidative burst ability was affected by weaning. We also wanted to know if any change in oxidative burst capacity of neutrophils was related to prenatal transportation stress and calf temperament.

## Materials and Methods

### *Experimental Design*

At weaning, bull calves (N = 24, PNS, N = 12, CONT, N = 12) were selected based on their prenatal treatment, weaning date and temperament scores as calculated from the average of their exit velocities and pen scores obtained at weaning. Selection of bulls included 3 calm PNS, 3 calm CONT, 3 temperamental PNS, and 3 temperamental CONT from each of the 2 weaning replicates. Blood samples were collected for CBC analysis, serum cortisol concentration, and neutrophil activity by generation of ROS production. From the same set of 24 calves, blood samples were obtained to isolate neutrophils to measure phorbol 12-myristate 13-acetate (PMA) induced ROS production at 0 d (N= 24), 2 d (N = 24), 6 d (N = 12), and 9 d (N = 12), post weaning. The CBCs were also taken during each of these times to correlate neutrophil blood cell counts.

### *Serum Collection*

Blood serum was collected via jugular vein puncture into a 15 mL red top tube (no additives). The serum was processed for cortisol concentrations. The blood was

refrigerated overnight at 4ºC and was centrifuged the next morning at 3,000 G for 20-25 min at 6-7˚C. Serum was flash frozen in liquid nitrogen and stored at -80˚C until being assayed.

*Cortisol Assay*

Serum concentrations of cortisol were determined in duplicate with the use of a single antibody radioimmunoassay (Coat-A-Count Cortisol Kit # TKC05, Siemens Medical Solutions Diagnostics, USA) that utilized rabbit anti-cortisol antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009; Hulbert et al., 2012). Serum concentrations of cortisol were determined by comparison of unknown samples with a standard curve that was generated with known concentrations of cortisol using Assay Zap software (Biosoft, Cambridge, UK). The minimum detectable cortisol concentrations for this assay were 1.2 ng/mL and the intra- and inter-assay coefficients of variation were 6.4 % and 5.6%, respectively. Data from this assay are presented as concentrations in ng/mL.

*CBC Testing*

Blood samples were collected in 2.5 mL EDTA tubes via jugular vein puncture at weaning (0 d) and processed by the IDEXX Protocyte Dx Hematology analyzer (IDEXX Laboratories Inc., Westbrook, ME). The samples were inverted a minimum of 15 times prior to being placed into the machine. The machine analyzed the sample and a report per calf sample was recorded. Additional CBC analysis of calf blood was done again on 2 d, 6 d, and 9 d post weaning. All samples were analyzed within 10 minutes of collection to reduce clotting, platelet aggregation, and any degradation to the samples.

The neutrophil:lymphocyte ratio (NL ratio) was calculated based upon the values obtained from the neutrophil and lymphocyte cell counts. The NL ratio is used as a measurement of stress assessment in animals (Widowski et al., 1989; Davis et al., 2008).

*Neutrophil Collection and Isolation*

Blood was collected via jugular vein puncture into acid citrate dextrose (ACD) tubes and transported on ice for 3 h from Overton, TX to Texas A&M University in College Station, TX where neutrophils were isolated in the laboratory and used in a reactive oxidation challenge. Neutrophils were isolated by differential centrifugation and hypotonic lysis of erythrocytes. After isolation, neutrophils were counted, and checked for cell viability on a cell counter. Neutrophil cell suspensions of 1 X $10^7$ cells/mL were made by dilution of the isolated neutrophils. An additional 20 µl of cell suspension was mixed with trypan blue and hand counted using a hemocytometer and a microscope. Estimates of cell viability were also quantified.

To measure the oxidative burst, we stimulated neutrophils with phorbol 12-myristate 13-acetate (PMA, Sigma, P1585-1 mg). The addition of PMA, the active component in croton oil and a known stimulator of oxidation in neutrophils, to the suspension media of neutrophils acts through the same pathway that would occur during neutrophil phagocytosis and can thus serve as a measure of the cells' phagocytic abilities (DeChatelet et al., 1976). Neutrophil cell suspensions of 1 X $10^7$ cells/mL were added to a 96-well plate along with 10 µL of 2',7'-dichlorofluorescin diacetate (DCFA, Sigma D6883-250 mg) probe and then stimulated with a concentration of 2.0 ng/µL of phorbol-12-myristate-13 acetate (PMA) in triplicate. The probe fluoresces in the presence of

ROS, allowing for measurement of neutrophil activity. The cells were incubated for 30 min, prior to quantification of fluorescence on a plate reader. Fluorescence was measured at 485/525 nm. Neutrophil isolations and their subsequent incubations were investigated at weaning (0 d) and again at 2 d, 6 d, and 9 d after weaning.

*Statistical Analysis*

The data were checked for normality using PROC univariate procedures of SAS during the preliminary analysis (SAS, 2011). Transformations were applied when needed in efforts to make the data more normally distributed. Subsequently, data were transformed by taking the natural log of the data. All statistical analysis was carried out on the transformed data in SAS software (SAS, 2011) using a mixed model with the fixed effects of d relative to weaning, weaning replicate, and prenatal treatment. Calf temperament score was also used as a covariate in the analysis. Where interactions and/or replicate were not found to be significant, it was taken out of the analysis. Following analysis, data were back calculated to obtain values reflective of the original data and are reported as the least squares mean ± standard deviations.

**Results**

*0 and 2 d for Both Weaning Replicates*

***CBCs***

The effect of weaning on immune cell indices, serum cortisol concentrations, and neutrophil ROS activity were investigated on the d of weaning (0 d) and 2 d post weaning in two weaning replicates. For the total WBCs, there was no effect of prenatal treatment ($P > 0.2$), although PNS calves (13.8 ± 0.85 K/μL) had numerically greater

WBCs than CONT calves (12.2 ± 0.85 K/μL). The d relative to weaning did not affect WBCs, as total cell counts were similar at 0 d (12.9 ± 0.62 K/μL) and 2 d (13.0 ± 0.62 K/μL); nor were there any interactions, ($P > 0.4$).

For the lymphocytes, prenatal treatment did not ($P > 0.1$) affect cell counts, however, PNS (7.8 ± 0.54 K/μL) calves had greater cell counts than CONT (6.7 ± 0.54 K/μL) calves. There was an effect of d relative to weaning ($P = 0.0002$), on the lymphocytes, with cell counts decreasing 2 d after weaning (Figure 36). No interactions were observed, ($P > 0.2$).

The monocytes were affected by d ($P < 0.0001$) with increases from 0 d to 2 d post weaning (Figure 37). A trend for treatment ($P = 0.0926$) to affect the monocyte cell counts was also observed, where PNS calves exhibited numerically greater cell counts than CONT calves (Figure 38). No interactions were observed to affect the monocytes, ($P > 0.9$).

There was no effect of treatment on the neutrophils, as PNS and CONT calves had neutrophil counts of 3.91 and 3.69 ± 1.08 K/μL, respectively. However, d relative to weaning ($P < 0.0001$) affected the number of neutrophils, as the stress of weaning led cell counts to increase at 2 d (Figure 39).

The eosinophils were not affected by weaning replicate ($P > 0.3$) or d relative to weaning ($P > 0.1$), but a replicate by d interaction ($P = 0.0062$) was observed. Replicate 1 had a decrease in eosinophils after weaning, and replicate 2 increased cell counts after weaning (Figure 40).

Basophils were not altered by prenatal treatment ($P > 0.8$), where the cell counts were 0.00084 and 0.0087 ± 0.0015 K/μL for CONT and PNS, respectively. There was an effect of d ($P = 0.0067$), with greater basophil numbers being observed at 2 d after weaning (Figure 41). No interactions ($P > 0.8$), were shown for the basophils.

The NL ratio differed by weaning replicate ($P = 0.0323$), as replicate 1 had a greater NL ratio than replicate 2 (Figure 42). There was also an effect of d ($P < 0.0001$), on the NL ratio, as greater ratios were seen on 2 d, compared to 0 d (Figure 43). There were no interactions ($P > 0.2$), nor was treatment a factor affecting the NL ratio, however, CONT (0.62 ± 0.03 K/μL) had numerically greater NL ratios than PNS (0.53 ± 0.03 K/μL) calves.

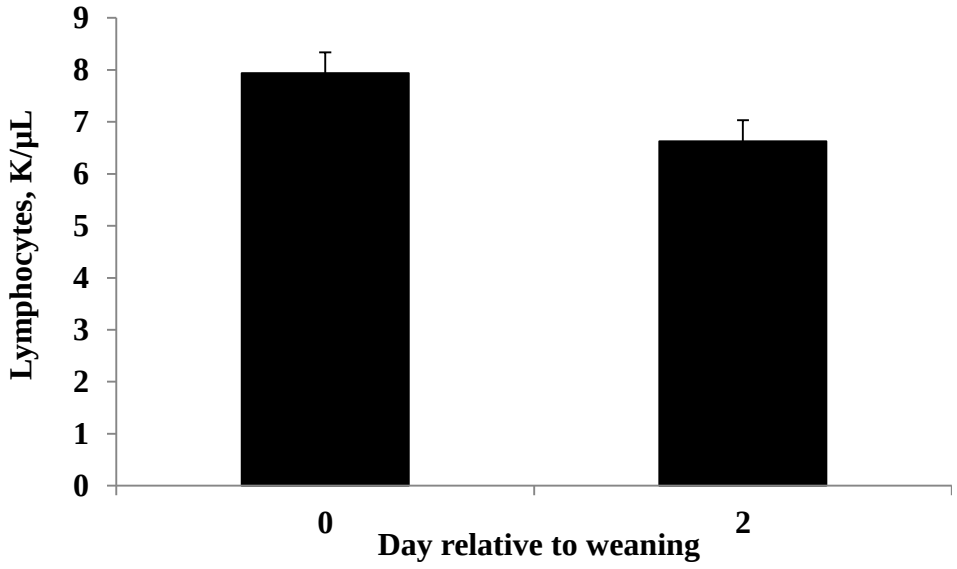


**Figure 36.** Calf lymphocyte cell counts in response to weaning. Lymphocyte numbers decreased after weaning ($P = 0.0002$) in all calves.

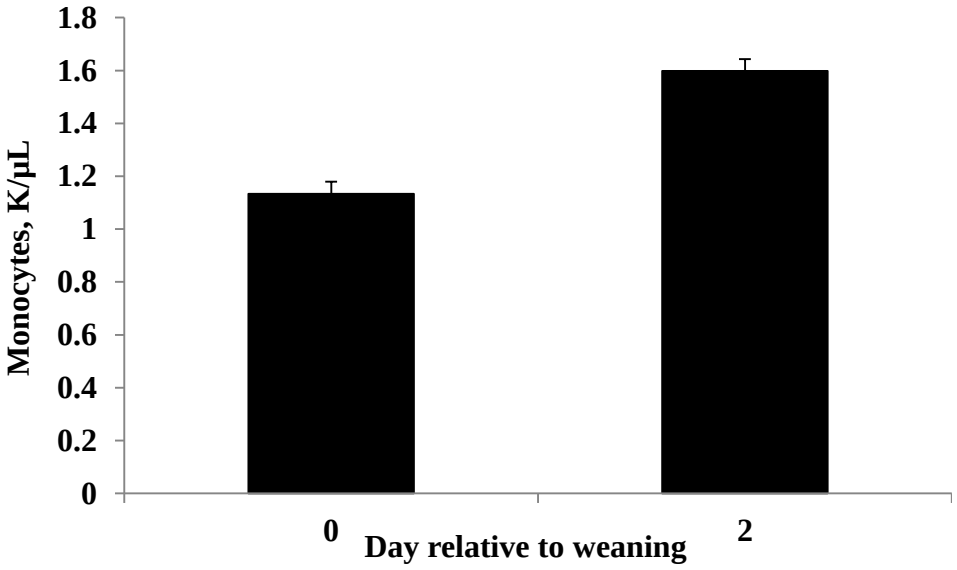


**Figure 37.** Calf monocyte cell counts in response to weaning. All calves displayed increased monocyte cell counts 2 d after weaning, ($P < 0.0001$).

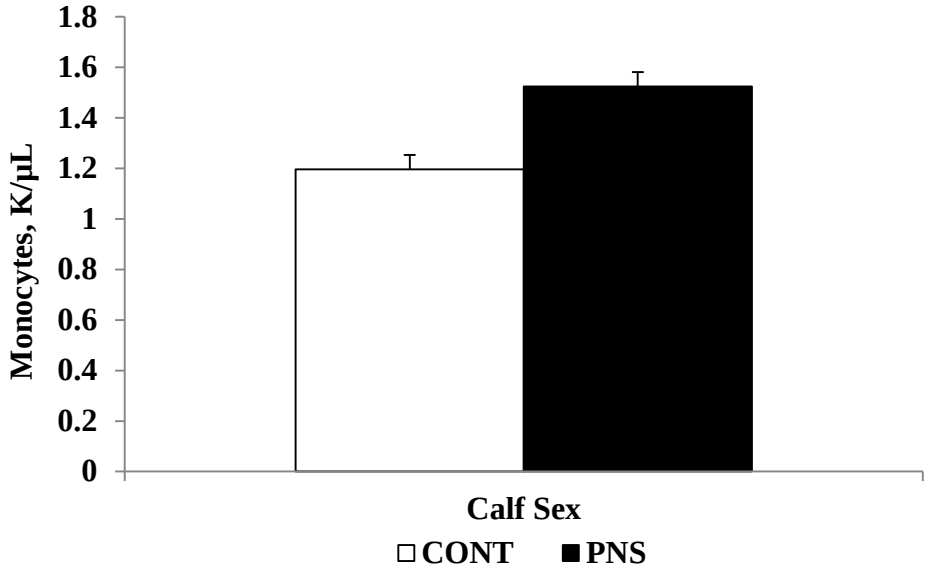


**Figure 38.** Calf monocyte cell counts by prenatal treatment in response to weaning. Prenatally stressed (PNS) calves tended ($P < 0.1$) to exhibit greater cell counts than control (CONT) calves in response to weaning.

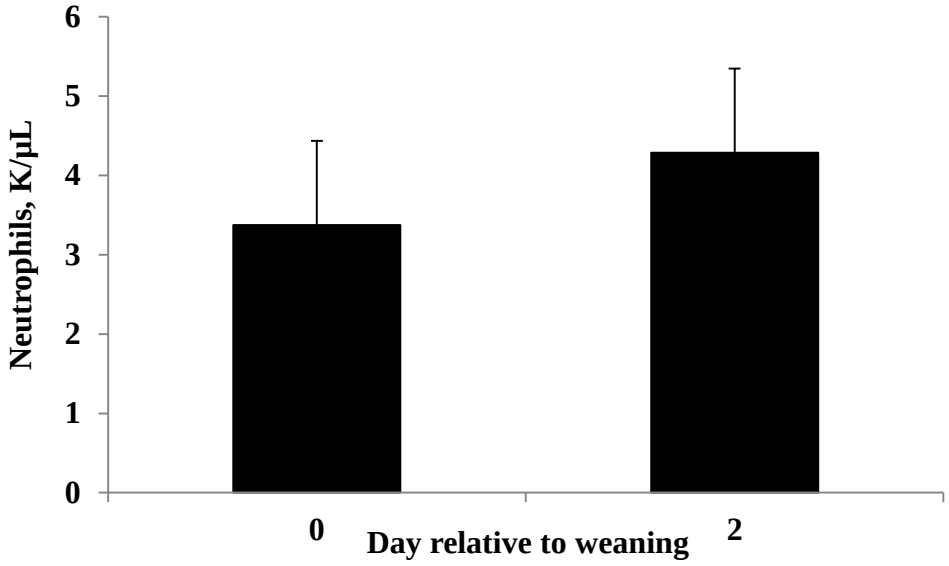


**Figure 39.** Calf neutrophil cell counts in response to weaning. The neutrophil cell count increased in response to the stress of weaning, ($P < 0.0001$).

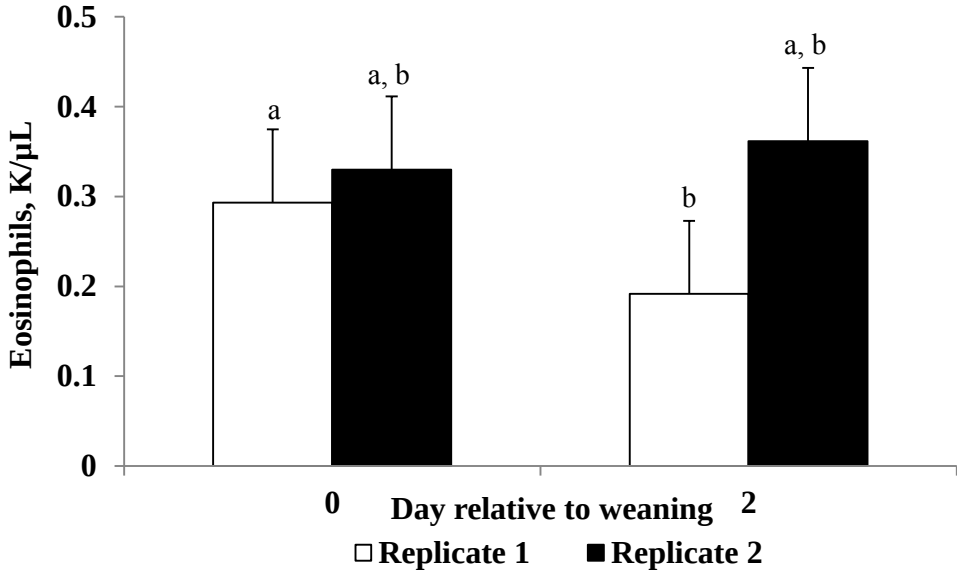


**Figure 40.** Calf eosinophil cell counts by replicate in response to weaning. Weaning replicate 1 exhibited a decrease in eosinophil cell counts, while in contrast replicate 2 exhibited an increase in cell counts, ($P = 0.0062$). Bars separated by different letters significantly differed in their means, ($P < 0.05$).

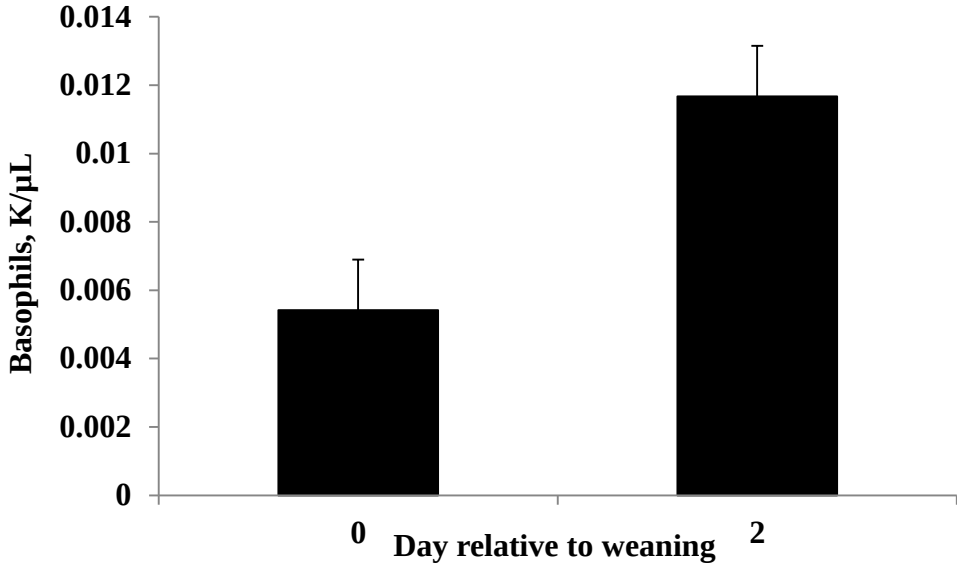


**Figure 41.** Calf basophil cell counts in response to weaning. Basophil numbers increased at 2 d post weaning, ($P = 0.0067$).

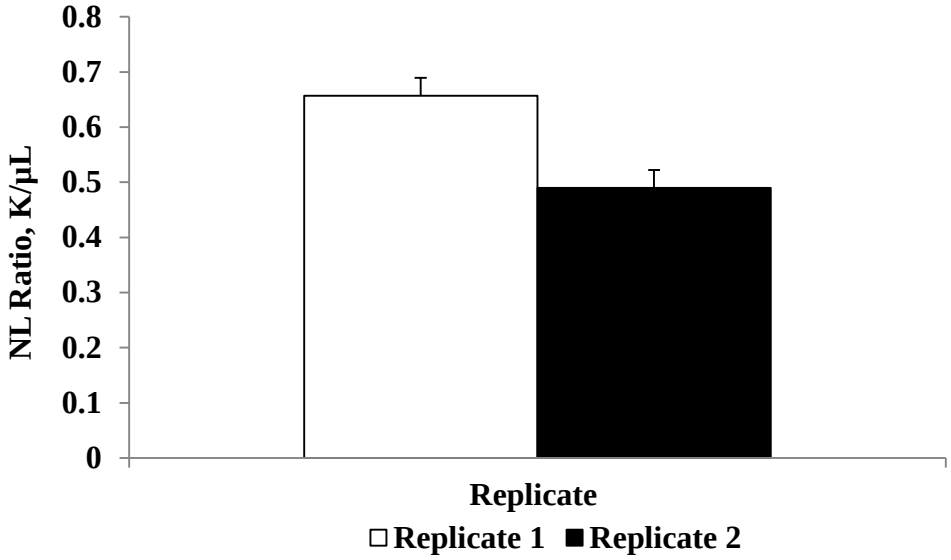


**Figure 42.** The calf neutrophil:lymphocyte (NL) ratio in response to weaning by weaning replicate. The weaning replicate affected the NL ratio, with replicate 1, having the greater NL ratio than replicate 2 ($P = 0.0323$).

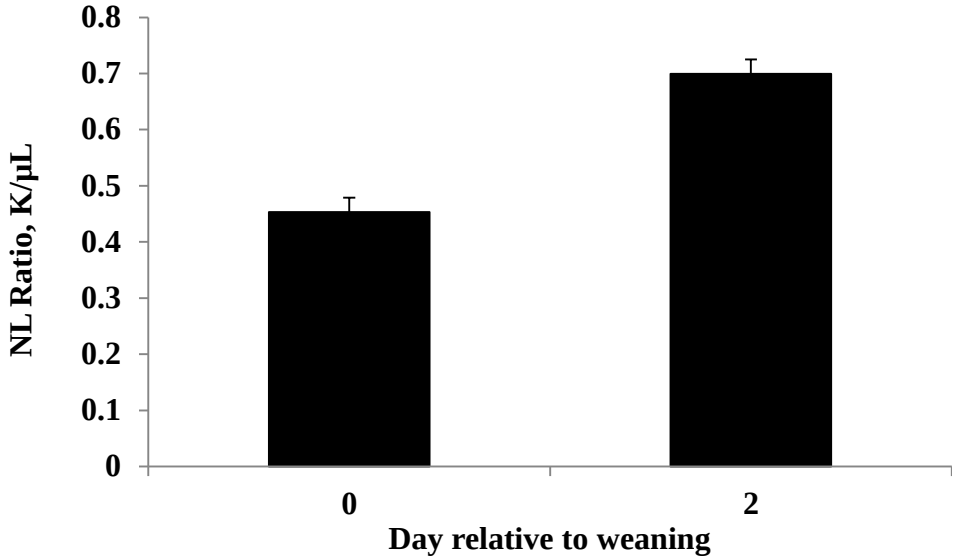


**Figure 43.** Calf neutrophil:lymphocyte (NL) in response to weaning. The NL ratio changed in response to the stress of weaning ($P < 0.0001$), with an increase in the ratio on 2 d post weaning.

***Cortisol***

Cortisol concentrations were examined at weaning and at 2 d post weaning. There was no effect of prenatal treatment ($P > 0.9$), with similar cortisol concentrations between CONT (15.5 ± 1. 13 ng/mL) and PNS (15.7 ± 1.13 ng/mL) calves observed. All calves demonstrated a stress response to weaning, as day ($P < 0.0001$) affected the cortisol response (Figure 44). Cortisol was greater at 2 d than on 0 d. Additionally, temperament ($P = 0.0003$) of the calf accounted for a large portion of the variability in cortisol concentrations. The temperament regression coefficient was 1.33 ± 1.07 ng/mL.

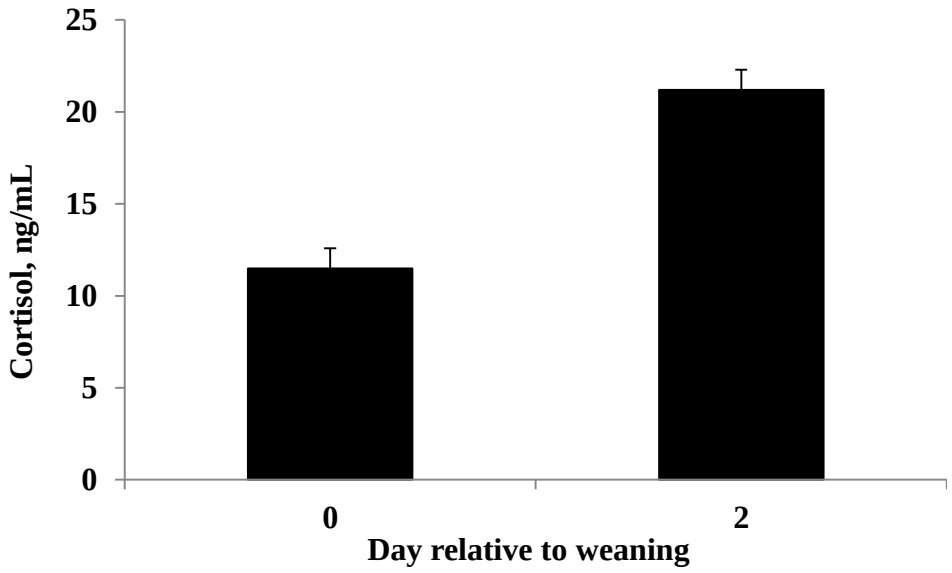


**Figure 44.** Calf cortisol concentrations in response to weaning. All calves demonstrated increased cortisol concentrations on 2 d in response to the stress of weaning ($P < 0.0001$). Calf temperament accounted for a significant amount of variation in cortisol concentrations, ($P = 0.0003$, temperament regression coefficient = 1.33 ± 1.07 ng/mL).

***ROS Activity***

The quantification of the capacity of the calves' neutrophils to release an oxidative burst provides observations into effects of prenatal stress on bull calves's neutrophils in response to weaning. It will also help to determine if there are changes in immune function that may affect disease susceptibility. The ROS analysis revealed no effect of weaning replicate alone ($P > 0.5$), with values of 1711 and 1498 ± 1.2 mean fluorescence units, being observed for replicate 1 and replicate 2, respectively. Nor was there an effect of prenatal treatment ($P > 0.4$), alone, with CONT and PNS calves having 1494 and 1716 ± 1.2 mean fluorescence units, respectively. The d relative to weaning affected calves' ROS ($P = 0.0002$), with a large decrease in ROS activity at 2 d post weaning (Figure 45) occurring in all calves. Additionally, d interacted with weaning replicate ($P = 0.0003$), where both replicates had similar ROS activity at 0 d, but at 2 d, replicate 2 showed a greater decrease in ROS activity compared to replicate 1 (Figure 46). Interestingly, an interaction between prenatal treatment and d relative to weaning also occurred, ($P = 0.0203$). The PNS calves had greater ROS than CONT at 0 d, and the CONT showed greater ROS activity than PNS on 2 d post weaning (Figure 47). However, the decrease in ROS activity observed was less in CONT compared to PNS calves.

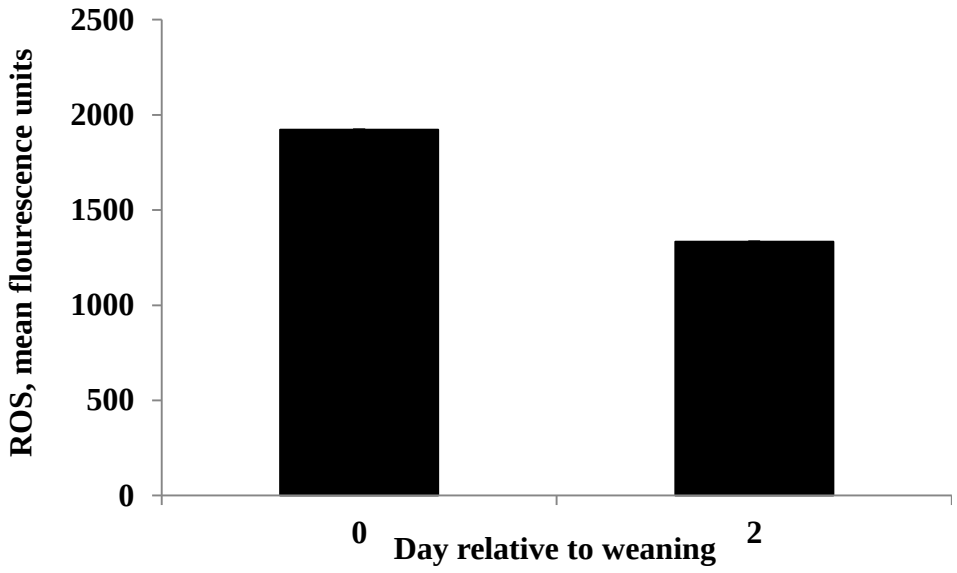


**Figure 45.** Calf neutrophil ROS activity in response to weaning. The d *(P* = 0.0002) relative to weaning affected ROS activity, with decreased ROS activity occurring in all calves 2 d after weaning. Error bars are included, however, due to low error values they may not be visible.

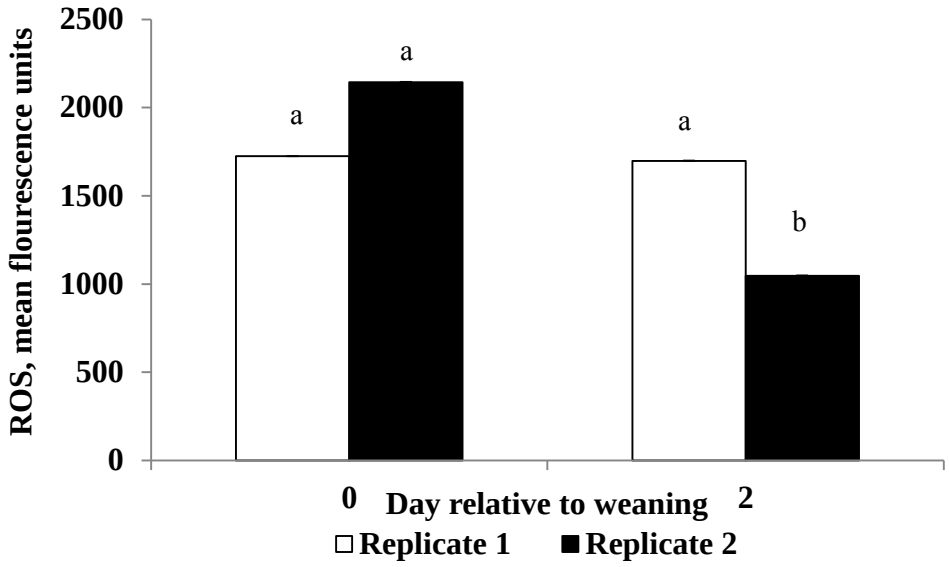


**Figure 46.** Calf weaning replicate and day relative to weaning interact to affect neutrophil ROS activity. Replicate 1 had a small decrease in response to weaning, while replicate 2 had a larger decrease in ROS activity on 2 d relative to weaning, ($P = 0.0003$). Bars separated by different letters differed in their means, ($P < 0.05$). Error bars are included, however due to low error values they may not be visible.

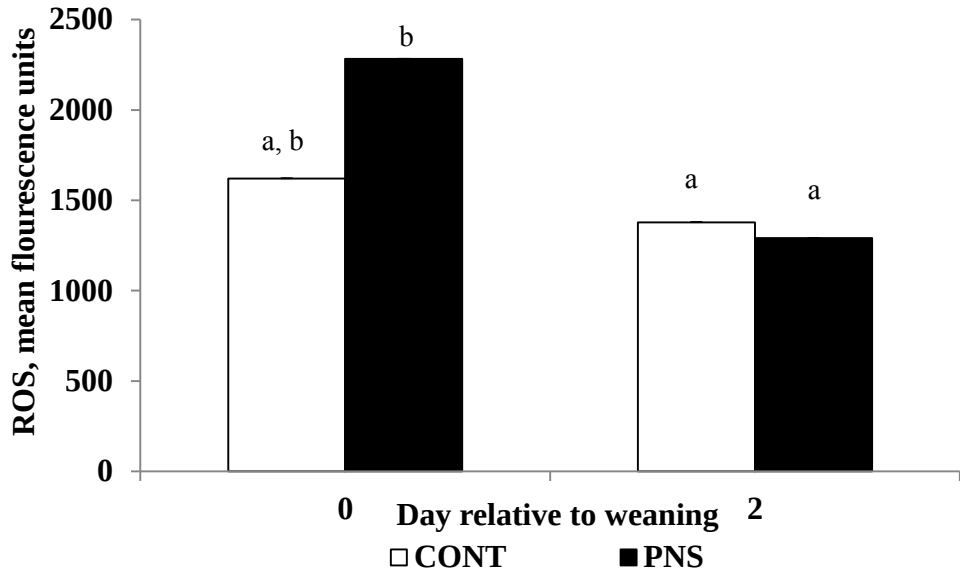


**Figure 47.** Prenatal treatment and day relative to weaning interact to affect calf neutrophil ROS activity. Control (CONT) calves had a smaller decrease in ROS activity than the prenatally stressed (PNS) calves, ($P = 0.0203$) on 2 d post weaning. Bars with different letters significantly differed in their means, ($P < 0.05$). Error bars are included, however due to low error values they may not be visible.

*0-9 d Relative to Weaning*

***Calf CBCs for Both Weaning Replicates***

The total WBCs were only affected by d relative to weaning ($P < 0.0001$), with similar cell counts seen on 0 and 2 d, followed by a decrease at 6 d and an increase at 9 d relative to weaning (Figure 48). There was no effect of prenatal treatment ($P > 0.1$), nor were there any interactions ($P > 0.4$), affecting the WBCs. Although treatment was not significant, the PNS calves (12.1 ± 1.07 K/μL) had numerically greater WBCs than CONT calves (10.7 ± 1.07 K/μL).

Observations of the lymphocytes showed d ($P < 0.0001$) to be the only factor affecting the cell counts, with decreases seen at 2 d and 6 d relative to weaning, followed by an increase on 9 d (Figure 49). No effects of treatment ($P > 0.1$) were observed as CONT and PNS calves had lymphocyte counts of 6.3 and 7.2 ± 0.4 K/μl, respectively. There were no interactions ($P > 0.3$) shown for the lymphocytes.

Evaluation of monocyte cell counts revealed d ($P < 0.0001$) to be a factor affecting their values, as the cell counts increased from 0 d to 2 d, followed by a decrease at 6 d and a small increase on 9 d (Figure 50). No effect of prenatal treatment ($P > 0.2$) was shown to affect the monocytes, although, PNS (1.36 ± 0.06 K/μL) had numerically greater cell counts than CONT (1.14 ± 0.06 K/μL) calves. Nor were there any interactions, ($P > 0.2$) affecting the monocyte cell counts.

For the neutrophils, prenatal treatment did not affect cell counts ($P >0.9$) as similar numbers were observed, 3.49 ± 0.4 and 3.45 ± 0.4 K/μL for CONT and PNS, respectively. However, the d relative to weaning had a large impact on cell counts ($P <$

0.0001), as values increased from 0 d to 2 d and then decreased at 6 d, followed by an increase on 9 d where they were close to values on reported 0 d (Figure 51). No interactions were observed for neutrophil counts, ($P > 0.3$).

The eosinophil cell counts were similarly only affected by d ($P < 0.0001$), with the greatest cell counts seen at weaning. The cell counts decreased through 6 d post weaning, before increasing slightly at 9 d post weaning (Figure 52). Prenatal stress did not affect eosinophil cell counts ($P > 0.5$), as 0.19 ± 0.05 K/μL and 0.23 ± 0.05 K/μL were seen for CONT and PNS calves, respectively. Nor were interactions observed, ($P > 0.9$) for the eosinophils.

Examination of the basophil cell counts revealed them to be affected by d relative to weaning ($P < 0.05$). Cell counts increased from 0 d to 2 d, followed by steady decreases through 9 d (Figure 53). Interestingly, temperament ($P = 0.0107$) was responsible for a large part of the variation in basophil cell counts, with a regression coefficient of 0.0016 ± 0.0006 K/μL. There was no effect of prenatal treatment ($P > 0.2$), with basophil counts of 0.009 and 0.007 ± 0.001 for CONT and PNS calves, respectively. Nor did any interactions exist for the basophils, ($P > 0.5$).

Upon analysis of the NL ratio, d ($P < 0.0001$) relative to weaning was shown to affect the ratio, with the greatest ratio observed on 2 d relative to weaning (Figure 54). The NL ratio was similar on 0 d, 6 d, and 9 d relative to weaning. Prenatal stress had no effect on the NL ratio ($P > 0.2$), with CONT (0.55 ± 0.03K/μL) and PNS (0.48 ± 0.03K/μL) having similar ratios. No interactions ($P > 0.7$) were observed either.

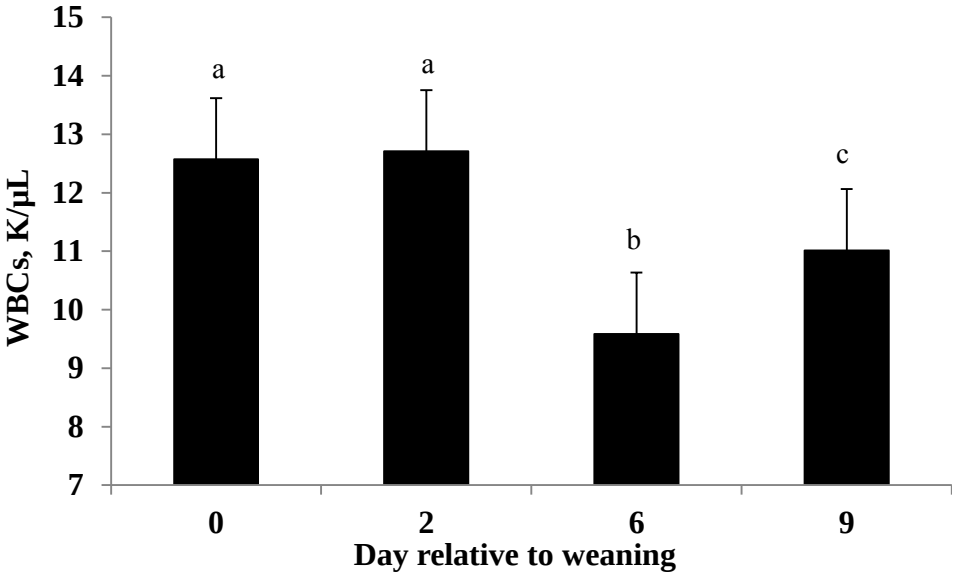


**Figure 48.** Calf WBCs from 0 d to 9 d post weaning. The cell counts were affected by day ($P < 0.0001$), with the greatest cell counts being seen on 0 d and 2 d, followed by decreases at 6 d. At 9 d, the WBCs increased, but had not reached the values they were on 0 d. Days with different letters significantly differed in their means, ($P < 0.05$).

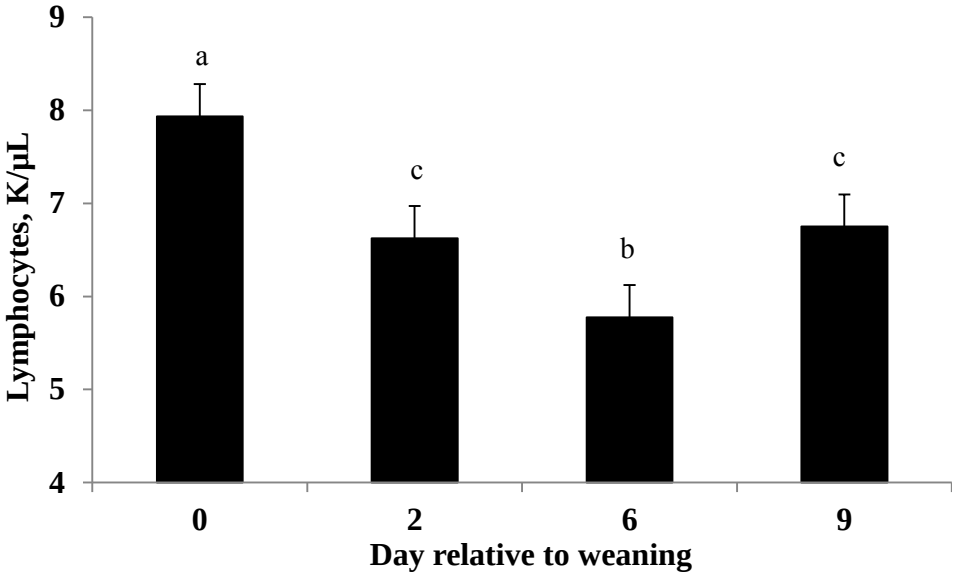


**Figure 49.** Calf lymphocyte cell counts from 0 d to 9 d post weaning. The lymphocytes decreased through 6 d after weaning, and increased slightly on 9 d, ($P < 0.0001$). Days separated by different letters significantly differed in their means, ($P < 0.05$).

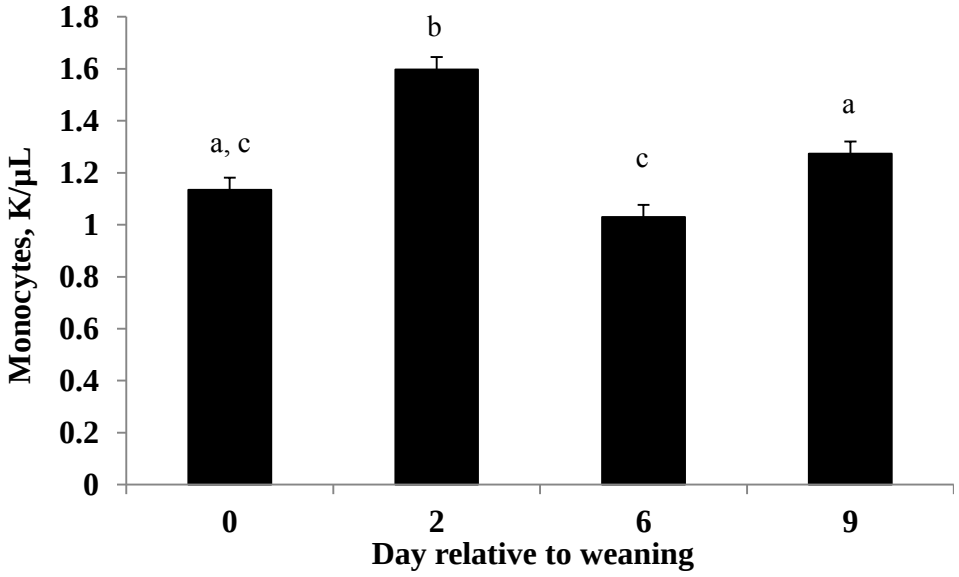


**Figure 50.** Calf monocyte cell counts from 0 d to 9 d post weaning. The cell counts changed with the day relative to weaning ($P < 0.0001$). Increased monocyte counts were observed on 2 d, followed by a decrease at 6 d, and a slight increase at 9 d. Days with different letters significantly differed in their means, ($P < 0.05$).

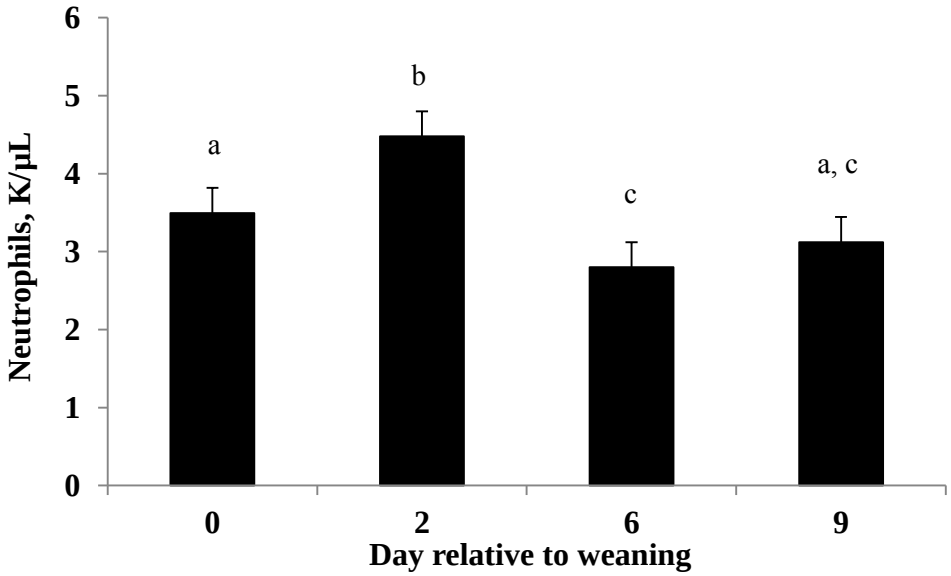


**Figure 51.** Calf neutrophil cell counts from 0 d to 9 d post weaning. The neutrophils increased in response to weaning, at 2 d, followed by a decrease at 6 d ($P < 0.0001$). Days separated by different letters significantly differed in their means, ($P < 0.05$).

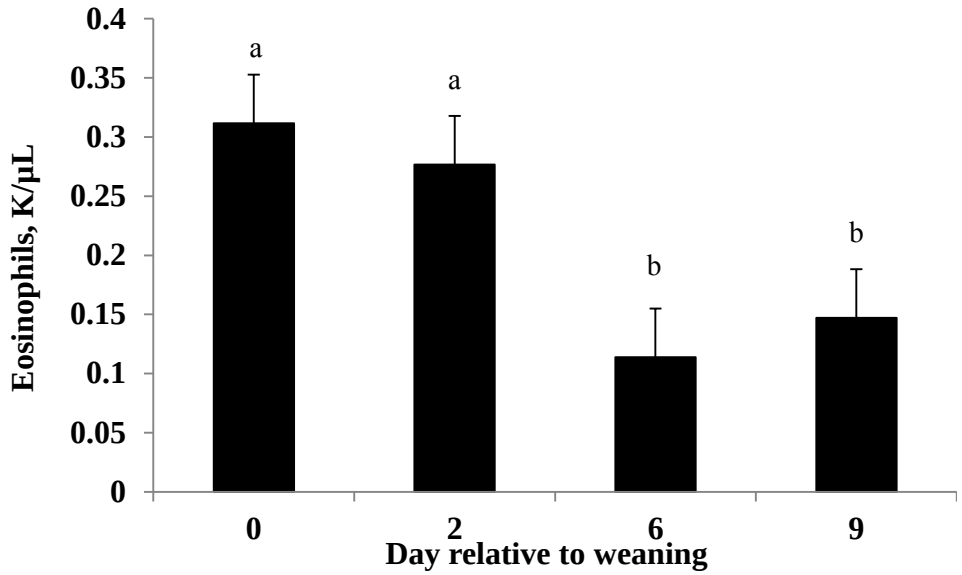


**Figure 52.** Calf eosinophil counts from 0 d to 9 d post weaning. The eosinophil counts were greatest at weaning and decreased through 6 d ($P < 0.0001$). Days separated by different letters significantly differed in their means, ($P < 0.05$).

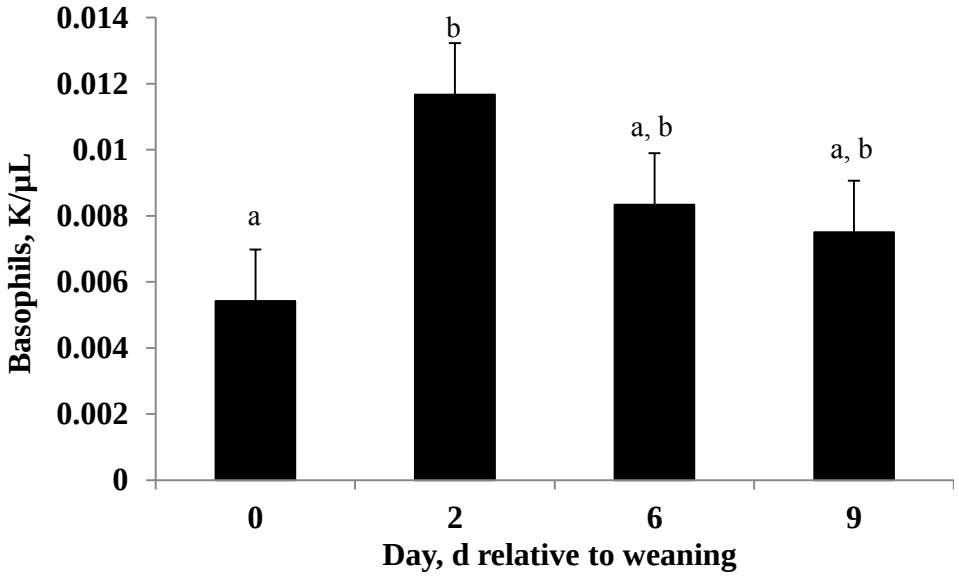


**Figure 53.** Calf basophil cell counts from 0 d to 9 d post weaning. The cell counts differed by day relative to weaning ($P < 0.05$). They increased at 2 d, and then steadily decreased through 9 d after weaning. Temperament of the calf accounted for much of the variation in basophil cell counts, ($P = 0.0107$, temperament regression coefficient = 0.0016 ± 0.0006 K/µL). Days separated by different letters significantly differed in their means, ($P < 0.05$).

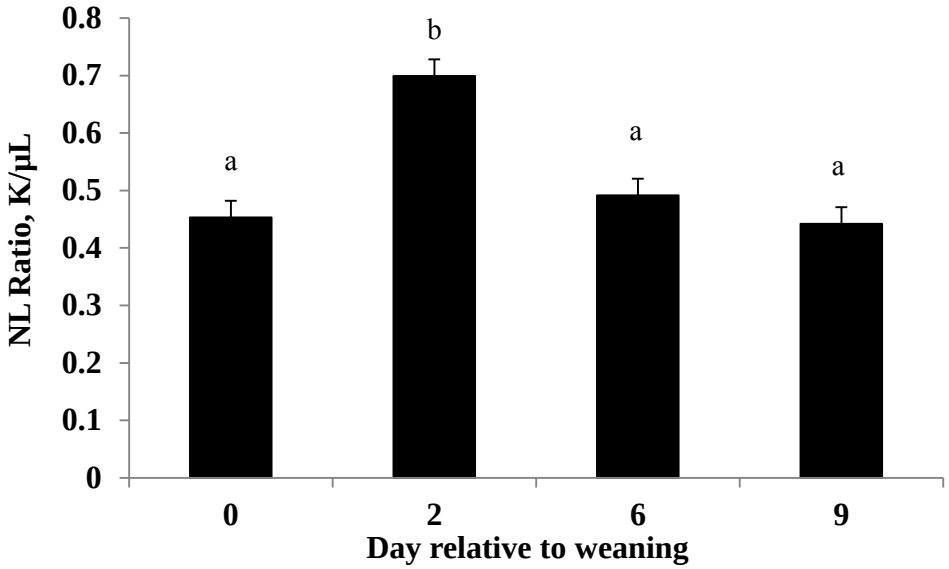


**Figure 54.** Calf neutrophil:lymphocyte (NL) ratio from 0 d to 9 d post weaning, (N = 24). The NL ratio was greatest on 2 d, and then decreased through 9 d to a similar value that was observed on 0 d. The d significantly affected the NL ratio, ($P < 0.0001$). Days separated by different letters significantly differed in their means, ($P < 0.05$).

***Cortisol 0-9 d for Weaning Replicate 2 Only***

Serum cortisol concentrations were analyzed from weaning replicate 2 (N = 12), at 0, 2, 6, and 9 d relative to weaning. The d ($P < 0.0001$) greatly affected the cortisol concentrations of all calves, with the greatest concentrations seen on 2 d, followed by a decrease in concentrations through 9 d (Figure 55). Although prenatal stress did not significantly ($P > 0.1$) affect cortisol concentrations, PNS (17.0 ± 1.17 ng/mL) calves had numerically greater cortisol concentrations than CONT (12.4 ± 1.17 ng/mL) calves. A large part of the variation in cortisol concentrations was attributed to calf temperament ($P = 0.0003$) with a regression coefficient of 1.32 ± 1.07 ng/mL. No interactions were observed for cortisol concentrations, ($P > 0.2$). However despite lack of significance, cortisol concentrations of PNS calves failed to return to their 0 d value by 9 d post weaning. Contrarily, CONT calves cortisol concentrations returned to their 0 d values by 9 d post weaning (Figure 56).

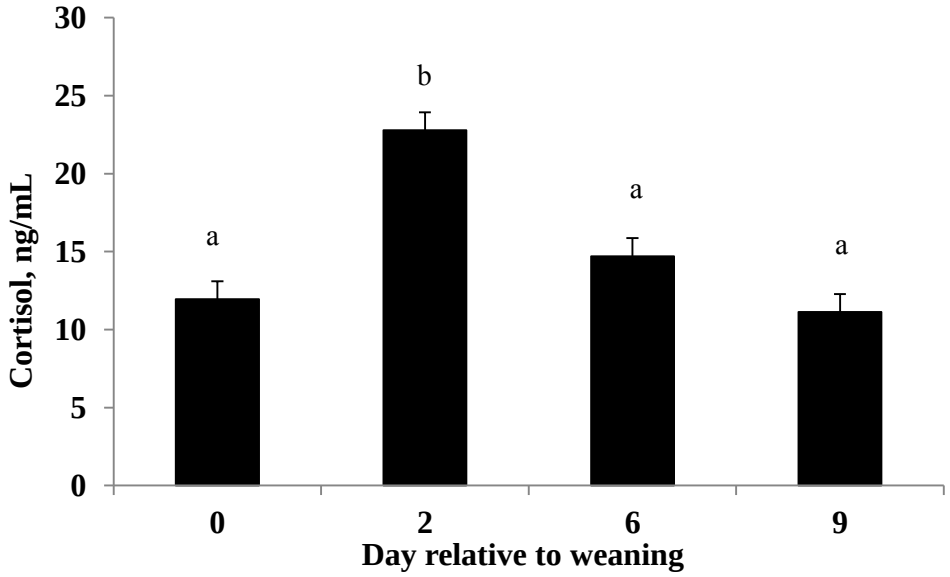


**Figure 55.** Replicate 2 calf cortisol concentrations in response to weaning, (N = 12). Cortisol concentrations peaked on 2 d as a result of the weaning stressor, and then steadily decreased back to pre-weaning values by 9 d ($P < 0.0001$). Temperament of the calf accounted for much of the variation in cortisol concentrations, ($P = 0.0003$, temperament regression coefficient = 1.32 ± 1.07 ng/mL). Days separated by different letters significantly differed in their means, ($P < 0.05$).

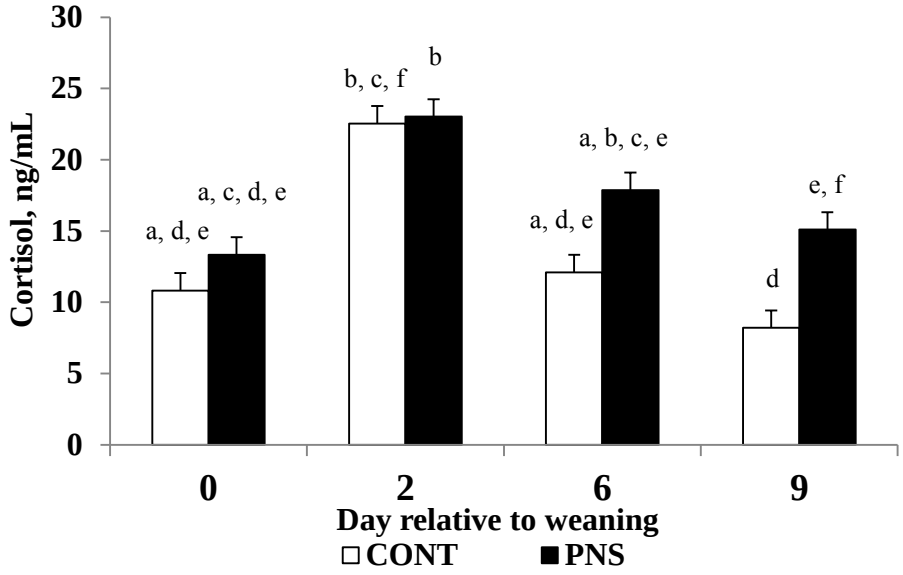


**Figure 56.** Cortisol concentrations in control (CONT, N = 6) versus prenatally stressed (PNS, N = 6) calves from 0 d to 9 d post weaning. The PNS calves still had numerically higher cortisol concentrations at 9 d relative to weaning, where CONT calves had cortisol concentrations that were even less than their 0 d values at 9 d, ($P > 0.2$). Bars separated by different letter significantly differed in their means, ($P < 0.05$).

***ROS Activity 0-9 d for Weaning Replicate 2 Only***

Isolated neutrophils were stimulated with PMA on 0, 2, 6, and 9 d relative to weaning to access their ability to produce ROS. Subsequent measurements of immunofluorescence were made. Neutrophil ROS activity differed by d ($P < 0.0001$) in relation to weaning, with neutrophils being less reactive on 2 d than on any of the other d in relation to weaning (Figure 57). There was no effect of prenatal stress alone ($P > 0.7$), as ROS activity was similar among CONT (1612 ± 1.2 mean fluorescence units) and PNS calves (1724 ± 1.2 mean fluorescence units). However, an interaction between prenatal treatment and d relative to weaning occurred ($P = 0.0118$), with PNS calves having greater ROS activity at 0 d, and a greater decrease in ROS activity on 2 d (Figure 58) compared to CONT calves. Additionally, by 9 d, the CONT calves had ROS values similar to their 0 d values, while PNS calves still had a lower production of ROS; showing PNS calves took longer to recover from the stress of weaning. Despite differences in ROS production, no differences based on prenatal treatment had been observed in the neutrophil cell counts. Interestingly, as the neutrophil counts increased in all calves at 2 d, the ROS activity in all calves decreased, (Figure 59, Figure 60).

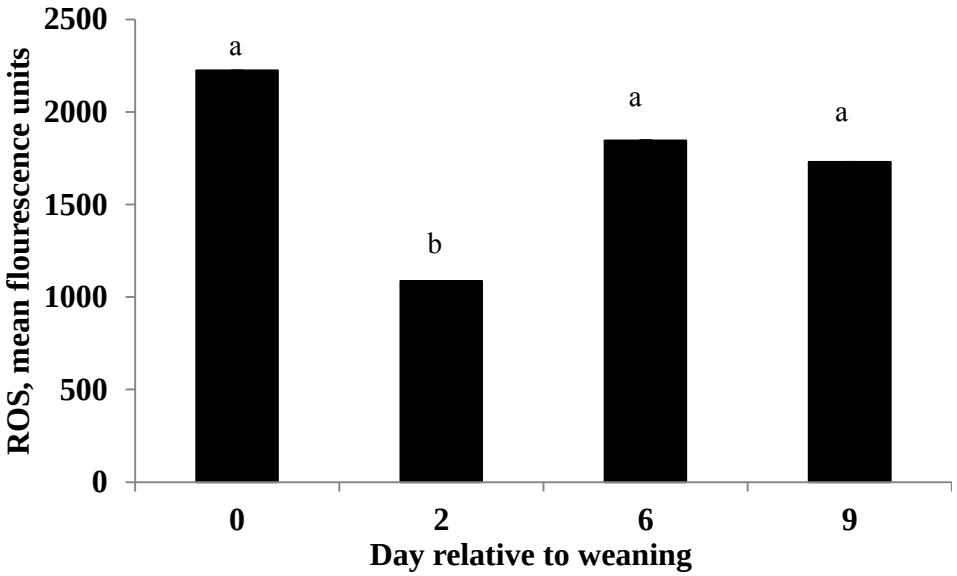


**Figure 57.** Calf neutrophil ROS activity from 0 d to 9 d post weaning. Neutrophil ROS activity was greatest on the day of weaning, and was least at 2 d relative to weaning ($P < 0.0001$). Days separated by different letters significantly differed in their means, ($P < 0.05$). Error bars are included, however due to low error values they may not be visible.

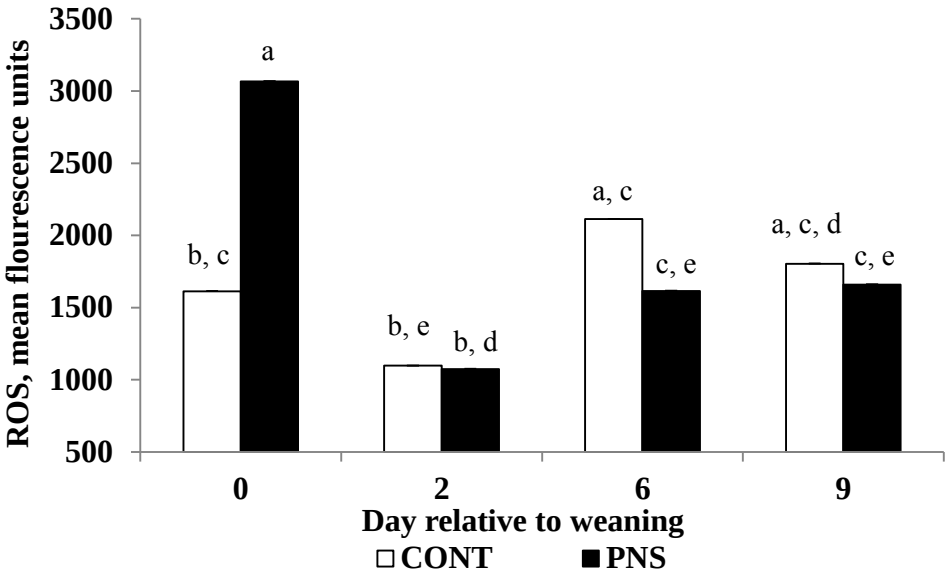


**Figure 58.** The effects of prenatal treatment and day relative to weaning on calf ROS activity. The control (CONT, N = 6) calves displayed less ROS activity on the day of weaning compared to prenatally stressed (PNS, N = 6) calves. However, PNS calves showed a greater change in ROS response from 0 d to 2 d, ($P = 0.0118$). Bars separated by different letters differed in their means, ($P < 0.05$). Error bars are included, however due to low error values they may not be visible.

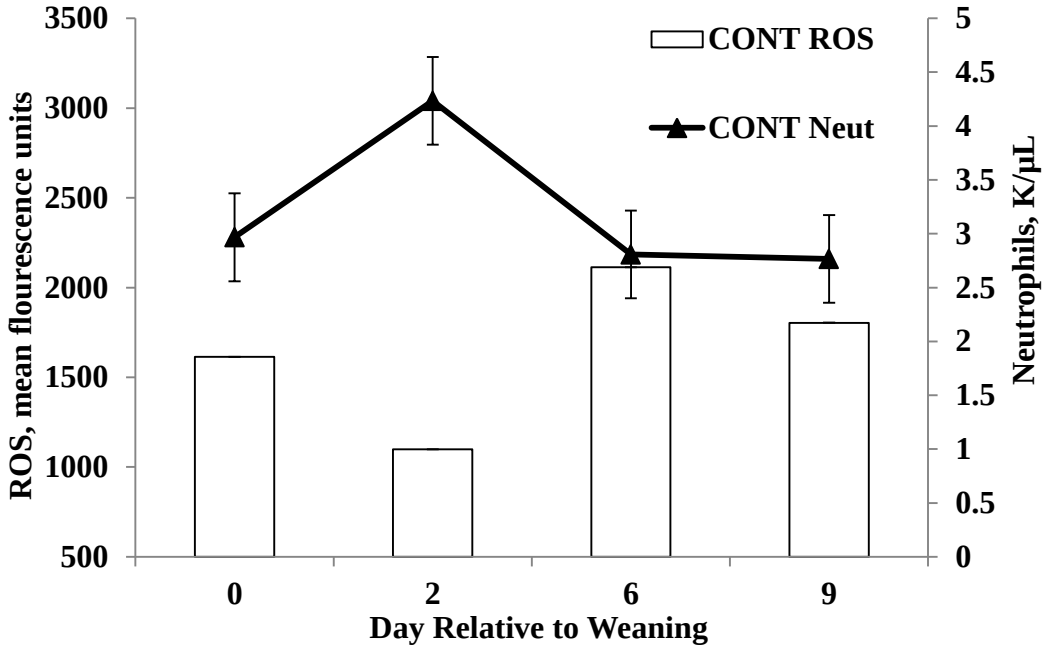


**Figure 59.** Effect of weaning on ROS activity versus neutrophil cell counts in control (CONT, N = 6) calves. As neutrophil counts increase at 2 d, ROS activity decreased. By 9 d post weaning, both the neutrophils and the ROS activity returned to their weaning day values.

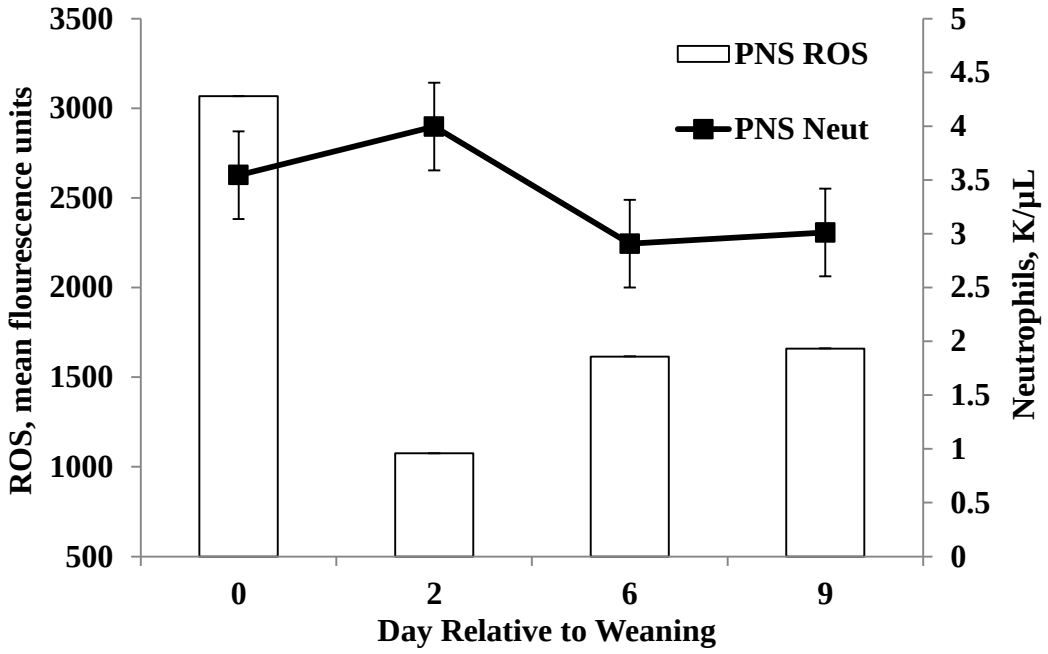


**Figure 60.** Effect of weaning on ROS activity versus neutrophil cell counts in prenatally stressed (PNS, N = 6) calves. At 2 d post weaning, ROS activity decreased while neutrophil cell counts increased. At 9 d post weaning, the neutrophil numbers had returned to their 0 d values, while ROS activity of the neutrophils was still reduced.

**Discussion**

Our data reaffirmed both endocrinologically and immunologically, that weaning is a stressful process for calves regardless of prenatal treatment. This is evidenced by the increases in cortisol concentrations and innate immune cell counts (with the exception of eosinophils which remained stable) coupled with decreases in adaptive immune indices seen on 2 d relative to weaning in all calves. Cortisol concentrations in the calves were elevated through 6 d post weaning and returned to 0 d values at 9 d relative to weaning. The prolonged elevations in cortisol are consistent with prior findings of increased cortisol concentrations through 7 d post weaning (Hickey et al., 2003). The total WBC numbers remained unchanged in response to weaning, due to the increase in neutrophils and simultaneous decrease in lymphocytes. This is further illustrated by the increased NL ratio that was reported.

The alterations in cortisol and immune indices we observed on d 2 correspond with other reports that most changes in response to weaning stress occur on 2 d post weaning. The increase in neutrophils along with the decrease in lymphocytes was consistent with prior studies on weaning stress in cattle (Hickey et al., 2003; Blanco et al., 2009; Lynch et al., 2010; Kim et al., 2011; O'Loughlin et al., 2012). Our observations on the increase in basophils and decrease in eosinophils also agree with prior data on weaning in beef calves (Phillips et al., 1989).

The increase in neutrophil counts demonstrates the transient neutrophilia which is known to occur in response to increased glucocorticoids and synthetic glucocorticoids, such as dexamethasone (Beveridge et al., 2008). Specifically, it is the down-regulation

of CD62L along with CD18, which contribute to the observed neutrophilia in the blood following a stressor or an increase in glucocorticoids (Burton et al., 1995; Tempelman et al., 2002). Neutrophilia results from the decreased L-selectin mRNA expression on blood neutrophils which is known to occur in various models of stress (Weber et al., 2004). Neutrophilia in response to acute stress represents the recruitment of mature cells from the bone marrow, while the decrease as observed in the case of lymphocytes is due to migration of the cells into various tissues (Dhabhar and McEwen, 1997; Dhabhar et al., 2012).

Glucocorticoids have been shown to decrease apoptosis in neutrophils and prolong their lifespan (Chang et al., 2004); further contributing to the increased number of circulating cells. Precisely, glucocorticoids acting via glucocorticoid receptors down regulate pro-apoptotic genes while up-regulating anti-apoptotic genes (Chang et al., 2004; Weber et al., 2007). Further support for glucocorticoid induced delays in apoptosis comes from Madsen-Bouterse et al., (2006), who demonstrated alterations in mitochondrial membrane stability and capsase-9 activation. Prolonging the lifespan of neutrophils may act to decrease their pro-inflammatory potential and increase their antimicrobial activities (Madsen-Bouterse et al., 2006; Weber et al., 2007). The rate of neutrophil apoptosis is believed to be related to the production of ROS, where high production of ROS leads to increased apoptosis and lower ROS production can decrease apoptosis (Ruiz et al., 2002).

Recent work has demonstrated several cytokine signaling pathways to be upregulated at 1 and 2 d post weaning in beef cattle. In particular these pathways

included those of CXCL5, CXCL7, CXCL8, CCL2, CCL24 and XCL2, all of which act as chemoattractants for both neutrophils and lymphocytes (O'Loughlin et al., 2012). Although we did not measure cytokines concentrations, it is likely that several of the aforementioned pathways were involved in regulating neutrophil trafficking and the resulting changes in neutrophil cell counts over the course of the study.

The monocyte cell counts increased at 2 d post weaning, which is contrary to the decrease in monocytes seen by O'Loughlin et al. (2012) on 7 d post weaning. However, monocytes become macrophages upon migration into tissues. There they are responsible for removing the debris of neutrophils which underwent apoptosis. It is possible the increase in monocytes may act to protect the animal from damage induced by increased neutrophil numbers. Additionally, macrophages act to activate the adaptive immune system through their production of cytokines and pro-inflammatory signals. Another reason for the disparity in monocyte cell counts from previous reports could be due to the taking of the blood sample itself, as this can be perceived as stressful to the animal. Epinephrine increases rapidly, even quicker than cortisol and is shown to recruit monocytes (Dhabhar et al., 2012).

We provided data showing neutrophil function decreases in response to weaning; despite increases in blood neutrophil cell counts on d 2. In other words, the neutrophils were less active on a per cell basis. The decrease in ROS agrees with prior reports of reduced phagocytosis and mean fluorescence in neutrophils of abruptly weaned calves (Lynch et al., 2010) and reductions in neutrophil ROS in response to sound stress in

male rats (Brown et al., 2008). Increased concentrations of glucocorticoids either endogenous or synthetic are known to interfere with phagocytic superoxide production.

The enzyme NADPH oxidase is responsible for the production of superoxide and modifications of its subunits are known to result in decreased ROS production (Babior, 1999; Sheppard et al., 2005). Gene expression studies demonstrated alterations in *phox* genes which make up subunits of the NADPH oxidase enzyme after cells were exposed to dexamethasone (Condino-Neto et al., 1998) and hydrocorticosterone (Ignacchiti et al., 2011). This led to reduced ROS production by the cells (Condino-Neto et al., 1998; Ignacchiti et al., 2011). Psychological stress before an examination was shown not only to decrease neutrophil ROS production but also to decrease p47-*phox* mRNA expression in the neutrophils (Ignacchiti et al., 2011). Although we did not investigate gene expression of the NADPH oxidase enzyme, it is likely the increased cortisol concentrations in our calves led to similar alterations in the calves' neutrophil NADPH oxidase enzyme complexes. This represents an area in need of future investigations.

Upon examination of the interaction between prenatal treatment and the day relative to weaning, the data showed that prenatally stressed calves displayed a greater decrease in their neutrophil ROS activity compared to CONT calves on 2 d post weaning. This was seen in both experimental replicates (0 and 2 d and the 0 through 9 d). The 0 d to 9 d weaning replicate provides evidence that the decrease in neutrophil function in the prenatally stressed calves has not fully recovered by 9 d post weaning, whereas CONT calves had ROS activity slightly greater than that observed at weaning. Similar results were seen for the PNS calves with regard to their cortisol concentrations.

The CONT calves' cortisol concentrations were even less than their 0 d values at 9 d relative to weaning, while PNS calves still had numerically greater cortisol concentrations at 9 d relative to their 0 d concentrations.

The initial decrease in ROS seen on 2 d post weaning may be viewed as protective as it would reduce the damage that may be done to the animal's tissues by increased neutrophil numbers. However, where neutrophil cell counts returned to their pre-weaning numbers by 9 d (or were slightly less); the ROS remained lower in PNS calves. Similar to the results of other studies (Barbazanges et al., 1996; Lay et al., 1997b; Götz et al., 2007) on the effects of prenatal stress, our prenatally stressed calves took longer to recover from a stressor compared to CONT calves, demonstrating impairment in their physiological stress response.

The differences observed in neutrophil function regarding the weaning replicates, may be attributed to the differences in age of the calves in each weaning replicate. The calves in replicate 2 were younger than the calves in replicate 1. Moreover, they demonstrated a smaller decrement to their immune function. This would lead us to conclude that weaning at an older age may be more beneficial in terms of immune response and protection from pathogens at weaning. Although, it would cost a producer more money to feed their animals for a longer amount of time, this investment may be supported by increased weaning weights and increased immune protection from pathogens that may be encountered during the process of weaning.

The process of weaning is considered a psychological stressor, and recent work in humans has supported our findings where exposure to a brief psychological stressor

(approximately 1 min) led to decreased ROS production when cells were stimulated by PMA (Shelton-Rayner et al., 2011).

We have provided some of the first evidence that prenatal stress can translate to differences in immune indices and immune function through the age of weaning in cattle. Gene expression experiments are warranted to determine if the differences in ROS production in prenatally stressed calves are due to epigenetic changes that may have been induced *in utero*. Additional research should consider the abilities of these calves to respond to future stressors and immune challenges both *in vivo* and *in vitro*. Also, investigations into their reproductive capabilities should be explored.

# CHAPTER V

# GENERAL DISCUSSION

## Summary/Conclusions

Prenatal and neonatal stressors have been associated with inappropriate behavior, diminished cognitive ability and susceptibility to pathogens, but the mechanisms remain unclear. Recently, an increasing amount of research on prenatal stress has been conducted in laboratory animals with relevance to human medicine. Despite this, only a handful of studies have been conducted to date on the consequences of prenatal stress in livestock, and even fewer if one considers its impact on offspring immune functions.

Through the series of experiments we conducted, we have validated repeated transportation of pregnant cows as a useful model for the investigation of prenatal stress on immune function in the prenatally stressed offspring of cattle. By employing this model of stress, we demonstrated the stress of transportation as it relates to the temperament of the cows. After parturition, we evaluated immune cell parameters from birth through the age of weaning and immune function with respect to neutrophil ROS at the age of weaning in prenatally stressed calves. Additionally, we examined cortisol responses of these calves from birth through weaning.

During the application of the prenatal stressor, all cows responded to each instance of transportation with increases in vaginal temperatures and cortisol concentrations. Importantly, the repeated transportation of pregnant cows yielded

variations in physiological stress responsiveness due to the cow's temperament. Temperamental cows had increased cortisol and glucose responses to handling and to transport compared to calm and intermediate cows. Similarly, we showed temperamental cows have the largest peaks in vaginal temperatures during transport. No differences in NEFA concentrations were discovered for any of the cows as result of the transportation. We showed all cows were able to habituate to the transportation stressor over time; however, the ability to habituate also relied on temperament, with temperamental cows being the slowest to habituate to the stressor.

Examination of the calves upon birth was the next step in the investigation. We were interested in determining if the stress responses exhibited by the pregnant cows (the increased cortisol concentrations, Chapter II), yielded differences in immune cell counts relative to calves from control dams that remained in pastures throughout their pregnancy. Our findings revealed variations in immune cell counts due to calf sex, with females having greater cell counts than males. Specifically, it was the numbers of neutrophils and the NL ratio that were greater in females compared to males. With regard to cortisol concentrations, we described 10 ng/mL greater cortisol concentrations in prenatally stressed calves at birth and greater cortisol concentrations in females beginning at 14 d of age.

Once the calves reached the age of weaning we evaluated immune cell counts and neutrophil function in response to the stress of weaning in both control and prenatally stressed bull calves. Despite increased numbers of neutrophils in the blood at 2 d post weaning in all calves, we observed decreased ROS production. Control calves'

cortisol concentrations were lower at 9 d compared to 0 d and their ROS was slightly greater on 9 d relative to 0 d. However, the prenatally stressed calves still exhibited decreased ROS activity and greater cortisol concentrations at 9 d post weaning compared to their values at weaning. Our results support observations that prenatally stressed animals take longer to recover from a stressor than animals which did not endure stress during gestation.

From the experiments carried out we propose the following considerations be made when examining immune cell counts and functions of calves:

1) Factors that need to be included are age, gender, temperament, and any prenatal treatment or stress the mother endured during pregnancy.

2) Weaning is stressful and can lead to decreased immune function. However, the insult to the immune system may be exacerbated by prenatal stress.

3) Prenatally stressed calves display impairments in their abilities to respond to the stress of weaning.

Based on the data we collected, additional studies are warranted in order to elucidate the mechanisms behind the differences in immune cell counts and immune functions. Neutrophils are the first immunological responders to protect neonatal calves from respiratory and enteric pathogens which are major causes of morbidity and mortality. If prenatal stress alters the ability of neutrophils to respond to pathogens or bacteria it may affect calves' susceptibility to disease. The influence of prenatal stressors on growth, meat quality and reproduction are areas of importance for production animals and need to be addressed further. Examination of the hormonal,

behavioral, growth and immune parameters of neonatal calves born to cows transported at several key times during pregnancy will provide insights into the genetic and/or epigenetic effects of prenatal stress on postnatal health and performance. Moreover, the way these prenatally stressed calves are able to cope with future stressors needs to be clarified, especially since research in laboratory animals often yields conflicting results.

# NOMENCLATURE

| | |
|---|---|
| AcH | acetylcholine |
| ACTH | adrenocorticotropin releasing hormone |
| ADG | average daily gains |
| APCs | antigen presenting cells |
| AUC | area under the curve |
| BET | betamethasone |
| BCS | body condition score |
| BRD | bovine respiratory disease |
| CBC | complete blood count |
| CFU-S | colony-forming units-spleen |
| CGD | chronic granulomatous disease |
| CNS | central nervous system |
| CPM | counts per minute |
| Con A | Concanavalin-A |
| CONT | control group |
| CRH | corticotropin releasing hormone |
| CTL | cytotoxic T cells |
| d | day(s) |
| DCFA | 2',7'-dichlorofluorescin diacetate |
| DEX | dexamethasone |

| | |
|---|---|
| dL | deciliter |
| E | epinephrine |
| EDTA | ethylenediaminetetraacetic acid |
| EV | exit velocity |
| FAD | flavin adenine dinucleotide |
| g | grams |
| GC | glucocorticoids |
| GR | glucocorticoid receptor |
| h | hour(s) |
| HOCl | hypochlorous acid |
| HPAA | hypothalamic-pituitary-adrenal-axis |
| ICAM | intracellular adhesion molecules |
| IFN | interferon |
| Ig | immunoglobulin(s) |
| IL | interleukin |
| LFA-1 | lymphocyte function-associated antigen -1 |
| LPS | lipopolysaccharide |
| MHC | major histocompatibility complex |
| MPO | myeloperoxidase |
| MR | mineralcorticoid receptor |
| MS | mature temperament score |
| NADPH | nicotinamide adenine dinucleotide |

| | |
|---|---|
| | phosphate |
| NE | norepinephrine |
| NEFA | non-esterified fatty acids |
| NKCs | natural killer cells |
| NL | neutrophil to lymphocyte |
| PAMPs | pathogen associated molecular patterns |
| PHA | phytohemagglutinin |
| PMN | polymorphonuclear leukocytes |
| PNS | prenatal(ly) stress |
| POMC | proopiomelanocortin |
| PRRs | pathogen recognition receptors |
| PS | pen score |
| PSC | pluripotent stem cells |
| PVN | paraventricular nucleus |
| RBC | red blood cells |
| RIA | radioimmunoassay |
| RORγt | retinoic acid receptor related orphan receptor γt |
| ROS | reactive oxidative species |
| T-bet | T-box expressed within T cell |
| TH | helper T cells |
| TLR | toll-like receptors |

| | |
|---|---|
| TNF | transforming neurotrophic factor |
| TGF | transforming growth factor |
| TRANS | transported group |
| VP | vasopressin |
| VT | vaginal temperature(s) |
| WBC | white blood cells |

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APPENDIX A

## GLUCOSE COLORIMETRY PROCEDURES

WAKO Autokit Glucose, 439-90901

**Test Procedure:**

Wavelength: 505 nm[*1] Light path: 1 cm Temperature: 37º C

| Sample (S) | Standard (Std) | | Blank (BL) | |
|---|---|---|---|---|
| Pipette into a cuvette | | | | |
| Sample | (µL) | 6.7 | -- | *2 |
| Standard 1 or 2 | (mL) | -- | 0.02 | -- |
| Working Solution | (mL) | 3 | 3 | 3 |
| Mix well, incubate for 5 minutes and measure the absorbance of S ($A_s$) and Std ($A_{std}$) against Bl ($A_{bl}$) at 505 nm. | | | | |

1. Accurately pipette 0.02 mL of sample or standard into the 1.0 mL cuvettes (test tubes)

2. Add 3.0 mL of working solution.

3. Mix, incubate for 5 minutes and measure the absorbance of Sample ($A_s$) and Standard ($A_{std}$) against Blank ($A_{bl}$) at 505 nm.

*1 When measure with two wavelengths λ1/ λ2 = 505/600 nm

*2 The omission of 0.2 mL of water does not significantly affect the absorbance measured.

**Results:**

Concentrations are determined using the following equation as supplied by the kit protocol:

Glucose (mg/dL) = $A_S/A_{Std}$ x $C_{Std}$

$A_S$ = Absorbance of sample

$A_{Std}$ = Absorbance of Standard I or II

$C_{Std}$ = Concentration of Standard I or II in mg/dL

**Materials Supplied:**

1. Buffer Solution 2 x 150 mL

60 mmol/L Phosphate buffer (pH 7.1) containing 5.3 mmol/L Phenol. Store at 2-10ºC

2. Color Reagent (When reconstituted) 2 x for 150 mL

Contains 0.13 U/mL Mutarotase, 9.0 U/mL Glucose oxidase, 0.65 U/mL Peroxidase, 0.50 mmol/L 4-Aminoantipyrine, 2.7 Ascorbate oxidase. Store at 2-10ºC .

3. Standard Solution I 1 x 10 mL

Containing 200 mg/dL Glucose. Store at 2-10ºC .

4. Standard Solution II 1 x 10 mL

Containing 500 mg/dL Glucose. Store at 2-10ºC .

**Working Solution:**

Dissolve the entire contents of one bottle (for 150 mL) of Color Reagent in one bottle 150 mL of Buffer Solution. This solution is stable for one month at 2-10ºC.

**Materials Required But Not Supplied:**

1. Pipettes

2. Water bath that can hold constant 37ºC

3. Spectrophotometer

APPENDIX B

## Wako HR Series NEFA-HR(2) Protocol

**Procedures:**

1. Mix Color Reagent A (powder) with Solvent A (50 mL liquid) using the connecter provided. Invert several times until dissolved and store in glass vial. Use solution to wash powder off of cap.
2. Mix Color Reagent B (powder) with Solvent B (25 mL liquid) using the connecter provided. Invert several times until dissolved and store in glass vial. Use solution to wash powder off of cap.
    a. Color Reagent B may be difficult to get into solution. Be sure solution is well mixed and all powder is dissolved.
3. Dilute Standards in distilled water as described below in microcentrifuge tubes:

| **Standard** | **Amount Stock in µL (1 mEq/L stock)** | **Amount $H_2O$ in µL** | **Final Concentration (mEq/L)** |
|---|---|---|---|
| 1 | 400 | -- | 1 |
| 2 | 200 # 1 | 200 | 0.5 |
| 3 | 200 # 2 | 200 | 0.25 |
| 4 | 200 # 3 | 200 | 0.125 |
| 5 | 200 # 4 | 200 | 0.0625 |
| 6 | -- | 200 | 0 |

4. Turn on plate reader.
    a. If plate reader has incubating abilities, be sure incubator is turned on to 37$^{o}$C.
    b. If plate reader does not have incubating abilities, turn on or check to make sure your incubator is set at 37$^{o}$C.
5. Accurately pipette 5 µL of sample, each level of calibrator and into each well of a 96-well plate (Fisher Scientific, cat # 353915). Add 200 µL of Color Reagent A to each well using a matrix pipetman.
6. Tap plate to mix.
    a. If plate reader has incubating abilities, place plate in incubator and incubate for 5 minutes at 37$^{o}$C.
    b. If plate reader does not have incubating abilities, place plate in incubator for 5 minutes at 37$^{o}$C and then place in microplate reader.
7. After 5 minutes read the plate at 505nm (sub: 600 nm). Copy and paste absorbance data into 'NEFA Assay Template.'
8. Add 100 µL of Color Reagent B to each well using a matrix pipetman.
9. Tap plate to mix.
    a. If plate reader has incubating abilities, place plate in incubator and incubate for 5 minutes at 37$^{o}$C.
    b. If plate reader does not have incubating abilities, place plate in incubator for 5 minutes at 37$^{o}$C and then place in microplate reader.

10. After 5 minutes read the plate at 505nm (sub: 600 nm). Copy and paste absorbance data into 'NEFA Assay Template.' Use template to calculate standard curve and sample concentrations.

**Materials Required**

*Supplied by Kit:*

1. Color Reagent A – Cat # 999-34691

2. Solvent A – Cat # 995-34791

3. Color Reagent B – Cat # 991-34891

4. Solvent B – Cat # 993-35191

5. NEFA Standard Solution – Cat # 276-76491

*Not Supplied by Kit:*

1. Plate reader with incubating and timing feature and capable of reading absorbance at 505-600 nm.

2. Glass vial to store reagents until use.

3. Microcentrifuge tubes.

4. 96-well plate (Fisher Scientific, cat # 353915)

5. Distilled water.

6. Pipettes 25 µL, 50 µL

7. Repeat pipettes 5 µL

8. Matrix pipetman 100 µL, 200 µL

APPENDIX C

**Cortisol Radioimmunoassay**

**Siemens Coat-A-Count Cortisol Kit # TKC05**

Protocol:

1. If choosing to create additional standards for concentrations between those supplied, make them prior to starting.

Additional Standards created:

2.35 µg/dL – add 25 µL of the 4.7 µg/dL standard to 25 µL of distilled $H_2O$. Vortex briefly.

0.5 µg/dL – add 25 µL of the 1 µg/dL standard to 25 µL of distilled $H_2O$. Vortex briefly.

0.25 µg/dL – add 25 µL of the 0.5 µg/dL standard to 25 µL of distilled $H_2O$. Vortex briefly.

0.125 µg/dL – add 25 µL of the 0.25 µg/dL standard to 25 µL of distilled $H_2O$. Vortex briefly.

2. Label uncoated tubes for NSB and TC (total count) tubes. Label coated tubes for the remaining standards. Label coated tubes in duplicate for each unknown sample.

3. Pipet 25 µL of the zero standard into both the NSB and zero tubes. Pipet 25 µL of each remaining standard and the pool into their respective tubes. . Pipet 25 µL of each unknown into their respective tubes.

4. Add 1.0 mL of $^{125}I$ Cortisol to each tube. Do this under the fume hood. Vortex for 5 seconds. Do not allow more than 10 min to elapse before continuing to the next step.

5. Cover with foil and incubate for 45 minutes in a 37ºC waterbath.

6. Remove from waterbath and decant thoroughly using foam decanting racks. Do not decant the TC tubes. Allow tubes to drain for 3 min before striking on absorbant paper to absorb the rest of the moisture. Pat hard on paper to rid the tubes of any remaining droplets.

7. Place the tubes in a gamma counter for 2 min to be read.

**Materials Supplied by Kit:**

Antibody Coated Tubes (stable at 2-8ºC until expiration date on bag.)

$^{125}$I Cortisol (Iodinated cortisol)

6 standards of cortisol : 0 µg/dL (2.0mL)

1 µg/dL (1.0 mL)

4.7 µg/dL (1.0 mL)

10 µg/dL (1.0 mL)

20 µg/dL (1.0 mL)

49 µg/dL (1.0 mL)

**Materials Required but not Supplied by Kit:**

Plain 12 X 75 polypropylene test tubes for NSB and TC tubes

Test tube racks

Micropipets: 25µL and 1.0 mL and disposable pipet tips.

Repeat pipetter

Waterbath, capable of maintaining 37ºC.

Foam decanting rack

Serum Pool for use as a standard (either make one or buy it prior to experiment.)

Gamma Counter

APPENDIX D

## Bovine Blood PMN Isolation

**Blood Collection:**

Collect blood samples via jugular vein puncture into 9 ACD vacutainer tubes. Invert samples to mix blood after collection. Place samples on ice until processing.

**Isolation of neutrophils from whole blood:**

1. Mix each ACD tube by inversion.

2. Centrifuge for 25 min at 1000 x g @ 4ºC. Keep tubes on ice between steps.

3. Using a transfer pipet, pipette out and discard plasma, buffy coat, and top 1/3 of the red blood cells.

4. Combine 3 of the 2/3 RBC layer tubes into one 50 mL tubes (≈ 15 mL total volume). Create 2-3 50 mL tubes per calf.

5. Add 15 mL of sterile, ice cold water to each 50 mL tube. Mix by inversion for 20 sec to lyse RBC (25 mL serological pipette).

6. Add 3.75 mL of sterile 5X PBS to each 50 mL tube and mix by inversion. Place on ice until all tubes are finished (5 mL serological pipette).

7. Centrifuge for 10 min at 250 x g @ 4ºC.

8. Decant (may need to pipette) liquid to isolate the white pellet at bottom of tube.

9. Wash each pellet with 10 mL of sterile 1X PBS. Vortex briefly to mix, and centrifuge tubes for 3 min at 500 x g @ 4ºC (10 mL serological pipette).

10. Decant. Lyse remaining RBC by adding 8 mL of ice cold, sterile water to each tube

and mix by inversion for 20 sec (may need to vortex to loosen pellet). Add 2 mL of 5X PBS & mix by inversion. Centrifuge for 3 min at 500 x g @ 4ºC (10 mL serological pipette).

11. Decant supernatant. Wash each pellet with 10 mL of RPMI 1640 w/ 5% FBS, vortex to mix, and centrifuge for 3 min at 450 x g @ 4ºC (10 ml serological pipette).

12. Repeat wash.

13. Decant & resuspend cells in 2 mL of RPMI 1640 Cell Culture Media w/ 5% FBS.

**Determination of cell purity and viability**

The Coulter Cell Counter in the Cohen lab will be used to determine cell concentration and viability.

**Materials Needed:**

Acid citrate dextrose (ACD) anticoagulated vacuatiner tubes (Solution A, ref # 364606)

50 mL conical tubes

Refrigerated centrifuge

Disposable transfer pipettes

Pipets (1 mL to 25 mL with disposable tips)

RPMI 1640 w/ 5% FBS

1X PBS

5X PBS

Sterile water

Micropipettes with disposable tips

APPENDIX E

## Oxidative Burst Assay

### Materials

1. DCFDA Probe: 2',7'-dichlorofluorescin diacetate (Sigma, D6883-250 mg)
2. PMA: phorbol 12-myristate 13-acetate (Sigma, P1585-1 mg)
3. DMSO: dimethyl sulfoxide
4. DPBS: 1x (Gibco)
5. Alcohol: 200 proof
6. Glucose (MW 180.16)
7. 96-well black plate
8. Neutrophil cells: $1x10^7$ /mL

### Solution Preparation

1. Diluent is RPMI with 5% FBS

2. 95% alcohol: Add 5mL ultra pure water into 95 mL alcohol, mix completely.

### Stock Solution Preparation

1. PMA 0.5mg/mL:

   Add 2 mL DMSO in one vial of PMA (1 mg). Dissolve it completely. Aliquot 30 µL per tube, and store them at -20°C. The final concentration is 0.5 mg/mL (500 ng/µL).

### High concentration solution

Mix 22 µL of 500 ng/µL freezer stock PMA with 478 µL of RPMI with 5% FBS.

**Low concentration solution**

Mix 3 µL of 500 ng/µL freezer stock PMA with 27 µL of RPMI with 5%FBS to make diluted stock.

Use 27.5 µL of this diluted stock. Mix with 472.5 µL of RPMI with 5% FBS.

2. DCFDA Probe 1 mg/mL:

Weigh 10 mg DCFDA into the tube with 10 mL 95% alcohol, dissolve it completely by vortexing. Then aliquot 500 µL per tube, keep them at -20°C. The final concentration is 1 mg/mL.

**Plate Setup:**

**Negative control well**

1. Add 50 µL neutrophil cells ($1x10^7$/mL) to each well.
2. Add 50 µL of RPMI with 5% FBS to each well.
3. Add 10 µL of probe to each well, the final volume in each well is 110 µL.

**High concentration well**

1. Add 50 µL neutrophil cells ($1x10^7$ /mL) to each well.
2. Add 40 µL of RPMI with 5% FBS to each well.
3. Add 10 µL of High concentration PMA to each well.
4. Add 10 µL of probe to each well, the final volume in each well is 110 µL.

**Low concentration well**

1. Add 50 µL neutrophil cells ($1x10^7$ /mL) to each well.
2. Add 40 µL of RPMI with 5% FBS to each well.
3. Add 10 µL of Low concentration PMA to each well.
4. Add 10 µL of probe to each well, the final volume in each well is 110 µL.

**Incubate:**

1. Cover the plate with foil. Incubate for 30 minutes at RT.
2. Read the plate at 485/528 nm on a plate reader.